

AIDS and illiberal measures

John Madday

Personal liberty should be protected in the battle against AIDS but not at all costs. Registration of infected individuals makes little sense but the testing of anonymous blood samples should proceed.

It was always on the cards that panic about the spread of AIDS (acquired immune deficiency syndrome) would engender illiberal adaptations of social principles long regarded as essential safeguards of personal liberty. As long as there is neither cure nor prophylactic for the disease, and while it remains possible that all those who become infected will eventually die (it is too soon to know what the proportions are), people are right to be alarmed that whole populations are at risk. The questions that arise are those familiar during wars: what restrictions of liberty are necessary to win and at what point, along that road, does illiberality make victory hollow?

Governments' first reactions (most creditable, so far, in Britain) have rightly been that public education must be the first line of defence. The obvious difficulty is that there is no record of success in changing people's sexual habits by exhortation and every reason to fear that the social groups that are both most vulnerable and most effective as agents of the spread of infection (young people chief among them) will be immune from persuasion. Another is that the sexual explicitness essential if public education is to be effective is itself socially disturbing. Already, in the United States, the Public Health Service and the Department of Education have been at odds (*Nature* 325, 287; 1987), while church groups in North America and Western Europe, including those that have made chastity a profession, are predictably beginning to dig in their heels. So people are beginning to cast around for other things to do.

The proposition that found its way onto last November's ballot in California (and which was thrown out) would simply have required that the condition of those known to be infected with the AIDS virus should be publicly registered as such. During the past weeks, similar ideas have been widely canvassed in West Germany (see page 650). On the face of things, they have a beguiling logic. If the threat to society is as serious as it could be, would it not make sense that those capable of transmitting the disease should in principle be identifiable and thus avoidable by those, for example, who might be about to go to bed with them? Unfortunately for the logic, there is not much chance that intending partners overtaken by sexual emergency would be diligent at consulting public registers, which would instead be used for other purposes, not all of them socially benign. The only enduring consequence of registration would be general ostracism of the infected, the principle of the old leper colonies. Would it make sense to make such a big leap backwards when other more seemly remedies are not exhausted?

There is, moreover, no good reason for believing that AIDS registration would be useful for any of the practical purposes for which it is at present canvassed. The claim that registration, even if it were only to take the form of a card carried by each individual proving that he or she had been tested for AIDS, would enable those at risk to avoid dangerous sexual partners, is largely denied by the circumstances of the case. The only tests for AIDS at present widely applicable are tests for antibody to the virus, which makes its appearance only some time after infection, perhaps a few months on the average.

None of this is an argument against testing. Especially as programmes of public education take hold in people's minds,

those who think they have been running risk will look for means of telling what the truth is. They will discover that reassurance is hard to come by. Testing facilities are mostly inadequate. Moreover, as the old-fashioned venereal-disease clinics know too well, there are awesome problems of confidentiality. Obviously the person who seeks testing must be told the result, but should the testers also have powers to trace and find all sexual contacts? On the face of things, the universal adoption of such a policy would make good sense. But there are horrendous difficulties. First, there is the obvious problem that those at risk are less likely to offer themselves for testing if they know their contacts will be traced. Then there is the difficulty of conducting an investigation of a person's life without casting a slur over all those concerned. There is also the problem that the tracing and testing of all sexual contacts of an infected person is expensive both of people's time and of money, especially if it is accompanied by proper attention to the counselling of those concerned. At some stage in this battle, it may seem that the compulsory tracing of the sexual contacts of infected people would be worthwhile; but nobody should expect too much of it. Meanwhile, a more subtle moral issue has arisen, both in Britain and the United States (where public health authorities have been beating their breasts on the subject for the past few weeks). The question is whether it can ever be proper to attempt to estimate the incidence of infection in the population at large, a number crucial to serious attempts to chart the future of the disease, by the anonymous testing of blood taken from patients for quite different purposes. A group of physicians in Britain has been asking for months for this course to be followed with blood sampled during pregnancies.

Anonymous testing would at present be disallowed because it violates the cornerstone of codes of ethics which lay down that people should be asked to give their consent to what is intended. Another ethical objection to the procedure is that the anonymous people who were found to be infected could not be given this information, even if they would find it valuable. These are issues on which governments and professional bodies should be prepared to bend the present rules. Nobody whose blood was tested anonymously would be the worse off, whatever the result of the test. Moreover, the data that could come from testing of this kind would be an invaluable means of monitoring the progress of the disease. If the battle against AIDS were a real war, there would be no doubt of what should be done. □

Where to go next?

John Madday

The House of Lords has told more truth than most on UK science: now it has another chance.

Is the quality of research too important to be left to politicians and civil servants? And is the dreadful condition into which the British research enterprise has been driven in the past seven years the result of the way in which government departments have been free to do what seems to them to be sensible, unhindered by protests from people in a position to know what damage their decisions would cause? These are the sceptical ques-



tions which have been implicit in the modest stream of constructive criticism which has come, during this unhappy period, from the only consistent source of commentary on the condition of the British research enterprise — the House of Lords Select Committee on Science and Technology. From the outset, in a report published in 1981, the committee has been pleading for a mechanism by which science and technology, in their own right and not as ingredients of some department's larger programme, should have some kind of political representation in the machinery of government. Last year (see *Nature* 325, 95; 1987) the committee repeated its demand in an even more persuasive form: not merely should there be a minister (other than the Prime Minister, who has a science degree) but also an advisory council including independent members that would have a say in the direction of events. The members of the committee, and the House of Lords in general, will have a chance further to pursue this cause in the debate arranged for Thursday of this week. They should push hard, but prudently (which is their habit).

Much of what has damaged British science in the past fifteen years or so has been done in the name of good sense. In the old days, two decades ago, there were no difficulties in the allocation of resources (for technical people knew what the needs were), but even the governments of those halcyon days (and their successors) would have benefitted from a means of knowing whether the funds spent so generously were also spent wisely and effectively. When it first became apparent (in the early 1970s) that it would not be possible for budgets to keep on rising, but that they might even have to shrink, those concerned did not understand the system well enough to know what should be done. It seemed sensible when those research councils whose prime function is the support of basic science responded to the prospect of stagnation by creating common facilities in principle accessible to all academic scientists; these same institutions, fifteen years later, are a large part of the reason why the support of creative people has become inflexible. Similarly, with the recent need, in Britain's serious economic condition, to cut back on public spending, it made sense for research to be put on the auction block. Ironically, the government's proper wish that, in the interests of good management, government departments should be responsible for their own spending meant that decisions about what to cut were left to people even less able to understand the difficulties. The way that the Ministry of Agriculture, Fisheries and Food has, during this period, played ducks and drakes with the research council responsible for agriculture is nothing but a scandal.

The lessons to be learned from this unhappy period are that politicians and civil servants have jointly ruined a previously splendid enterprise for the lack of a framework of policy within which to carry out their well-intentioned but often mistaken decisions. So much can be told from the way that government departments have recurrently been swept by fashions — such as the fad for information technology and 'relevance' — and have sought to attain worthy objectives by methods fated to be ineffectual, attempting to skew the pattern of research while neglecting the need for mechanisms to ensure that useful outcomes can be successfully applied. At the same time, important ingredients of the equation "research success = prosperity" have been left untouched — British military research remains too far apart from the rest of the British enterprise. During this whole period, these errors of commission and omission would have been less easily foisted on the system if there had been a formal (if small) part of the machinery of government with the authority to question political decisions touching science and its application. For the advice to be that of an independent council would have the further benefit of helping to bring important issues out into the open and of providing a measure of continuity conspicuous by its absence in the management of British research. The House of Lords report had much else to say last year, but this is the nub of its demand. Can the government say no yet again? □

Shades of green and blue

The hostile reception to UK government plans to promote other uses of farmland is undeserved.

WITH one wave of a £25 million wand, Britain's Minister of Agriculture, Mr Michael Jopling last week tried to turn beef into buildings and butter into beech woods. So began what is sure to become a growing campaign on the ground in Britain, as opposed to in the corridors of Brussels, to shrink the food mountains of Europe. An edifice to the sluggishness of European politics, these mountains have continued to grow at the expense of the taxpayers who heavily subsidize farmers to produce more food than Europe can consume. Since there cannot be a British taxpayer who whole-heartedly approves of this, unless he is also a farmer, Mr Jopling (who happens to be a farmer) must have hoped for a generally favourable response to his plans to entice farmers, by means of money, to find alternative uses for some of their less-good land. Why, instead, was his wand-waving greeted with the kind of reception that is usually reserved for the wicked fairy in the Christmas pantomime?

One justifiable reason would have been that the alternatives were too limited. But they are not. Of the £25 million, £10 million is to be offered to persuade farmers to turn grassland into woodland, £7 million to pay them not to harm so-called Environmentally Sensitive Areas, and smaller sums to encourage forestry as well as farmhouse industries such as cheese-making and providing clotted cream teas for tourists. Moreover, except for the very best of land, the constraints on converting it to non-agricultural use are to be relaxed — that is, if the local planning authorities see fit to allow a mediocre field to be turned into a golf course or a cluster of 'exclusive georgian-style residence', they need no longer take into account the loss of potential beef, butter or milk.

Farmers, however, can smell a rat a mile off. While having no objection to the prospect of selling at great profit a field or two to a property developer, they know that in general the changes are a harbinger of much worse to come from a government set on reducing public expenditure in general and farming subsidies in particular. Nor were those whose prime concern is for the environment to be found rooting for the minister. This being an election year, Mr Jopling had clearly hoped to gain 'green votes' by some of his measures. For example, by doubling the budget available for paying farmers to stick to traditional methods in Environmentally Sensitive Areas, it will be possible to ensure the protection of some more areas from the waiting list. And one cry of all environmentalists has been at least partly heeded by the promise that a certain proportion, perhaps 20 per cent, of any woodland planted under the proposed scheme of additional subsidy will have to consist of broad-leaved trees. Where Mr Jopling lost the 'green vote' — and is in danger also of losing the 'blue vote' of traditional Conservative voters from commuter belts — is in relaxing the restrictions on non-agricultural use of farmland, particularly with the ensuing prospect of urban sprawl.

It is possible to feel some sympathy for Mr Jopling. After being careful to feed the barracudas before dipping his toe in the water, he still found them hungry enough to attack. He and his counterpart in the Department of the Environment are now going to have to consider how best to proceed. The Environment Secretary would be wise to say more on how planning authorities are to interpret the relaxation of the agricultural factor in considering applications for redevelopment of farming land. After all, he is the final court of appeal for developers denied permission. But that apart, the best course of action of the government is to stand firm and carefully monitor the results. For this is only the start of a programme that will, by some accounts, convert 30 per cent of English farmland to other uses by the end of this century. Viewed in that light, Mr Jopling's pilot experiment is likely to do more good than harm. □

World eavesdrops on the Moscow 'peace' conference

- Sakharov challenges both superpowers *LB*
- Gorbachev sounds a cautionary note

London

LAST weekend, an International Forum "for a non-nuclear world and the survival of humanity" took place in Moscow. Formally the 1,000 plus participants came as 'individuals', and the meeting had no connection with any government, including that of the Soviet Union. This did not inhibit Mr Mikhail Gorbachev from entertaining the delegates to a closing reception at the Kremlin, where he delivered a major speech, castigating the United States for "effectively trying to scrap the Anti-Ballistic Missile treaty of 1972" and warning that the Soviet Union would be resuming the testing of its own nuclear weapons. But the concept of individual participation did allow the organizers to invite Dr Andrei Sakharov to participate, thus attracting the attention of the world media.

Sakharov's presence was an entirely voluntary act. His personal campaign for nuclear disarmament goes back almost twenty years — indeed, he made his debut as a dissident with an essay on "progress, peaceful coexistence and intellectual freedom" which was triggered by his concern over the arms race. Moreover, his stance on the US Strategic Defense Initiative (SDI) was formulated in an article in *Foreign Affairs* in 1983: namely, that for such a system to operate as an adequate deterrent, it would have to achieve a degree of precision not feasible within the current or foreseeable state of the art. This same argument was advanced earlier this month by a spokesman for the Soviet government, Gennadii Gerasimov, who compared the degree of precision involved with that needed to produce a library of several thousand volumes without a single misprint. At this point Dr Sakharov's views coincide with those of the government. This may well have influenced those who decided to end his exile — although they do not challenge his credibility of independence of thought.

The only limit, as far as this particular forum was concerned, was the external one imposed by the Soviet media. For, in his address to the working group of scientists he criticized both superpowers, saying on the one hand that "the West must not try to corner the Soviet Union", for "a cornered nation is always dangerous", but on the other hand that a "more democratic and open society" in the Soviet Union (including the right to emigrate from it) would be a major safeguard of peace. The Soviet media, however, re-

ported only his criticisms of the Western stance and of SDI technology, ignoring his remarks about the Soviet Union.

Dr Sakharov's presence, while focusing world attention on the forum as a whole, nevertheless meant that some other issues were overlooked in official statements. (The sessions themselves were closed.) Nothing was publicly said, apparently, of the missing computer scientist Vladimir Aleksandrov, who worked on the Soviet computer model of a possible 'nuclear winter' and who vanished from a peace conference in Madrid in spring 1985, although his continuing absence is itself a source of international tension among his former colleagues. Nor was much notice paid by the West to the peace initiatives of the Bulgarian-launched 'Ecological Forum', several of whose leading members were present in Moscow. One of the movement's founders, Dr Nansen Bekhar, explained during a visit to Britain last month, that this body aims to be not simply a "professionals for peace" group,



Yelena Bonner and Andrei Sakharov in their Moscow home.

but to work actively for the elimination of environmental problems which can themselves provoke or exacerbate international tensions.

As far as the Moscow meeting was concerned, Dr Sakharov's rehabilitation seems to have been complete. He was present at the Kremlin reception where he warmly applauded Mr Gorbachev's statement of his commitment to "new approaches on humanitarian issues". But to those conference participants who attended the forum in the hopes of influencing Soviet policy, Mr Gorbachev sounded a warning — the new response to humanitarian issues was not, he said, a response to pressure from the West. This stance tallies with the statement by Central Committee member Georgii Arbatov last weekend that the release of the Jewish activist Iosif Begun was actually delayed by the Moscow protests on his behalf. The Soviet leaders, it would seem, are prepared to exercise a new clemency, but do not wish to be seen as acting under compulsion.

Vera Rich

Space station partnership still in jeopardy

Washington

INTERNATIONAL collaboration in the US space station remains in the balance, but less precariously than before last week's meeting of delegations from the US and potential partner governments. The statement issued after the meeting says that Canada, Japan and the members of the European Space Agency (ESA) will continue their negotiations with the United States towards a civil space station. But the National Aeronautics and Space Administration (NASA), the prime mover of the project, seems as worried by internal as by international threats to the project.

The objective of allowing the three non-American partners to satisfy themselves that the space station will not be dominated by the US military seems only partly to have been attained. The statement saying that negotiations are to continue gives no timetable, even though ESA, for one, needs to know where it stands by its next council meeting in June.

Observers who accept NASA's contention that it has never hidden the possibility of US military participation admit that some partners may have been shaken by the prospect of the space station being used for testing components of the Strategic Defense Initiative. The need now is for a form of words assuring non-military users that their projects will not be overridden by military needs while not usurping NASA's overall responsibility for managing the station. Part of the problem is that there are two agreements to be negotiated, one among the participating governments and one between NASA and other operating agencies such as ESA.

Both the programme office and NASA's comptroller have carried out reviews of the project, which have in turn been reviewed by Dr James Fletcher, NASA's administrator. Fletcher is now negotiating with the White House's Office of Management and Budget about the cost, believed to be much greater than the \$8,000 million originally forecast. Whatever the outcome, some delay seems unavoidable. By 1994, the date first fixed for full operation of the space station, the best that can be hoped for is the launching of some parts of it.

Terence Finn, a deputy director at NASA's space station office, says that the station could allow the United States to regain its leadership in space science. Accommodating the Pentagon's interests should be feasible but will require diplomacy, he says. He admits to worry that the Department of Defense might become "insensitive to the international and research flavor" of the space station. □

AIDS registration becoming a political issue in Germany *t102*

Hamburg

THE control of AIDS (acquired immune deficiency syndrome) is gaining political momentum daily and is now generating as much public anxiety as unemployment, foreign affairs, environment and tax. In the wake of last month's general election, the winners — the Christian Democrats (CDU), their sister party CSU and the Liberals (FDP) — have been negotiating to install a new Christian-Liberal coalition and have witnessed AIDS gaining political stature. Compulsory registration of AIDS victims is now a prime issue.

Franz-Josef Strauss, Minister President of the state of Bavaria and head of the CSU, has asked his partners to discuss the epidemiological aspect of the disease. He and his party demand a compulsory registration for all who are infected with human immunodeficiency viruses — similar to the strategy adopted in Sweden.

Newspapers daily report the disease in big headlines, mentioning new cases and speculating on the chances of infection. AIDS had been ignored for a long time by West German politicians. The Health Committee of the Bundestag (parliament) was briefed for the first time in autumn 1985. One year on, the first plenary discussion took place; it lasted only half an hour. The result was a commitment to research and public information.

The Minister of Health, Heiner Geissler, did almost nothing about AIDS during his period in the Cabinet. His successor, the Christian Democrat Rita Süssmuth, who came into office in September 1985, did not share Geissler's opinion that AIDS was a problem only of the high-risk groups. Her ministry started a campaign of enlightenment, paying millions of marks for advertising in newspapers and magazines, television, folders and



posters. A current youth centre poster declares, "Carnival will be over on Ash Wednesday, AIDS won't".

Süssmuth is an enthusiastic proponent of the use of condoms — she was photographed for the magazine *Der Spiegel* in a huge plastic bag. Her campaign is supported by the Liberals, Social Democrats and Greens. She rejects, however, compulsory registration, at least at the moment. Her political influence will very much depend on the success of her campaign. In Berlin the campaign has considerably reduced the number of cases of venereal disease. At the end of January there were 875 individuals in West Germany registered with AIDS, out of whom 409 have already died. Süssmuth estimates that the number of infected persons lies between 30,000 and 100,000 and that by 1990 half a million West Germans will have the virus.

CSU health expert Kurt Faltlhauser wrote a paper for the CSU members of parliament in Bonn in which he demanded compulsory AIDS tests for risk groups such as haemophiliacs, drug addicts and registered prostitutes, as well as for all pregnant women and for travellers and foreigners from "risk countries". A voluntary test (anonymous and free of costs) is also to be offered to every resident of West Germany.

At a later stage, the CSU expert recommends "compulsory tests and registration for the whole population", for which the preparations must be made now. Friedrich Zimmermann, CSU Minister of the Interior, even considers restrictions on infected persons. CSU MP Erich Riedl demands the separation of groups in places where people have to live together such as schools, clubs and factories.

The Liberals also demand an 'AIDS ministry'. Part of the Süssmuth Ministry and parts of the Ministry of Labour should be put together to create a new Health Ministry, which could better cope with the growing problem. **Jürgen Neffe**

MRC pushing for government funds for AIDS vaccine research

London

WITH an annual budget that would soon be £10 million, it would be possible to organize and direct an effective AIDS (acquired immune deficiency syndrome) vaccine research programme in the United Kingdom.

The claim is that of the Medical Research Council (MRC) which has devised the blueprint of such a plan, currently being studied by government. The disclosure was made last week to the House of Commons Social Services Committee as it took evidence from the MRC on AIDS.

The plan, submitted to government through Mr Kenneth Baker, Minister for Education and Science, at the end of December, outlines a strategy that will give the council extra AIDS funding of £2.5 million in the first year, rising to £4-6 million in the second and then £10 million before the fifth year.

In the written evidence to the committee the MRC had claimed: "We believe that the UK has the scientific capability for making a decisive contribution to the development of an AIDS vaccine provided that a political lead is given and that substantial extra funding, outside the Science Vote, is forthcoming. A number of distinguished scientists in the UK have expressed a wish to take part in a programme of research to develop an AIDS vaccine."

The paper, supplemented by oral evidence principally from Lord Jellicoe and Sir James Gowans, paints a grim picture.

To determine the behaviour of the disease, groups of infected individuals need to be studied for another 20 years or more, concludes the MRC. But a new British programme to find a vaccine should be mounted and the MRC would speedily allocate the funds through its mechanisms for 'special project grants'.

The MRC, which created a working party on AIDS about 4 years ago, has since provided eight special grants for AIDS research from its own funds and has diverted resources at its own establishment, the Clinical Research Centre at a total cost of £700,000 a year. Also seven 'special allocations' have been made for epidemiological studies, funded by the health departments to the tune of £300,000 a year.

The council was recently awarded an extra £1 million a year to pay for further AIDS research. Additional research in Africa, where the MRC already supports projects in The Gambia and Zambia, is now also expected to be financed.

The MRC evidence emphasized that general screening of the population for human immunodeficiency virus infection raises difficult ethical and legal questions. But it concludes that: "There is a need now both for extensive facilities for the voluntary testing of all sexually active members of the population who are willing to know if they are infected, and the testing of representative samples of the population to monitor the spread of infection". **Bill Johnstone**

Protein engineers to gain from telecommunications selloff

Tokyo

UNEXPECTEDLY, the privatization of Nippon Telegraph and Telephone (NTT) appears to have benefited Japan's molecular biologists. A small part of the massive funds flowing into the Japanese government coffers from the privatization will be channelled into the creation of a protein engineering research institute.

Last week NTT emerged on the Tokyo stock exchange as the largest private company in the world. Although sceptics considered the stock overvalued at ¥1.2 million (\$8,000) per share, trading in NTT shares was paralysed for two days as buyers outnumbered sellers by a hundred to one. Only when the government released a further 100,000 shares on the market did trading get under way at ¥1.6 million, and by the weekend the price had risen to nearly ¥1.8 million. If the price holds, the government stands to make over ¥13 million million from the sale of 50 per cent of NTT during the next three fiscal years.

While most of the money accruing from the privatization is expected to be swallowed by the government's huge fiscal deficit (debt servicing payments currently run at about ¥11 million million a year), a small portion is being directed into support for the Japan Key Technology Center, an organization for the promotion of basic research that was established jointly by the Ministry of International Trade and

group of advisors about suitable projects to establish in the field of molecular biology and biotechnology. The protein engineering institute was selected from a list of about twenty ideas drawn up by the group. Established as a joint-stock corporation in April last year, the institute will receive funds of around ¥17,000 million over the next ten years. Fourteen biotechnology companies, including the 'big five' (Kyowa Hakko Kogyo Co., Takeda Chemical Industries Ltd, Toray Industries Inc., Mitsubishi Chemical Industries and Ajinomoto Co.), will contribute 30 per cent of the funds, the rest coming from Japan Key Tech.

The institute, which has temporarily set up headquarters in Tokyo, will move next spring to the former site of EXPO 70 in Suita City near Osaka, an area already endowed with several biotechnology-related institutes. The land for a three-storey laboratory is being provided rent

free by the local Osaka government, which plans to build a bioscience institute next door, and it is expected that the Osaka government will take over the protein engineering institute when funding runs out in ten years.

Professor Morio Ikehara, managing director of the institute, plans to install the latest high-tech equipment, including a supercomputer and the world's most powerful nuclear magnetic resonance spectrometer (600 MHz). There will be five departments — structural analysis of proteins, correlation between structure and function, production (genetic engineering), purification plus evaluation of functions, and a database and computer service department. But projects will be drawn from every department to encourage interdisciplinary research.

But at present a battle is raging between those on the management side of industry and the academics over organization and staffing of the institute. The academics are seeking talent with ideas, whereas those in industry want good management and a quick return on investment.

David Swinbanks

Interest in the human genome project reaches new heights

Washington

THE effort to map and ultimately to sequence the human genome has captured the attention of the White House. The cabinet level Domestic Policy Council (DPC) has asked its working group on biotechnology to prepare a report on the human genome project. A first instalment of the report, due next month, will detail present US activities. Future instalments could include a framework for coordinating federal activities on the project.

The working group will meet on 23 February to decide how best to proceed. According to David Kingsbury, its chairman, the report will probably be written by a subgroup on which the National Institutes of Health (NIH), the departments of Energy and State and the White House Office of Science and Technology Policy are expected to be represented.

The first report will be primarily to educate DPC members on the costs and benefits of pursuing the genome project. The notion that such an endeavour could cost \$3,000 million attracted White House attention. No federal agency is contemplating such a large project, but several are already working on parts of a sequencing and mapping project.

The plans of the Department of Energy (DoE) for the human genome project are the best defined. It is supporting the development of a set of DNA libraries specific to individual chromosomes. Work is under way at Los Alamos National Labor-

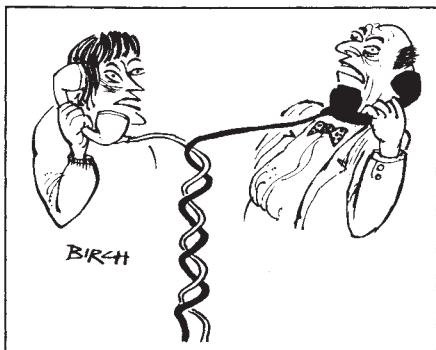
atory on chromosome 16 and at Lawrence Livermore National Laboratory on chromosome 19. A consortium headed by Cassandra Smith at Columbia University is working on chromosomes 21 and X.

Charles DeLisi, who heads DoE's efforts, says that the department will spend approximately \$5 million on the human genome project in this fiscal year and \$11.5 million next year. As well as the DNA libraries, DoE will focus on new sequencing technology and computational activities. DeLisi says that techniques for calculating structure from sequences will become increasingly important. An advisory group met earlier this month to specify plans for this year's DoE activities.

NIH, which supports several projects that will be relevant to any mapping and sequencing effort, has not yet decided exactly what its role in the human genome project will be. NIH director James Wyngaarden chairs a small working group that will guide NIH directions on the project. George Palade of Yale University serves as the group's external advisor.

Other reports on the human genome project are in the offing. The National Academy of Sciences Board on Basic Biology plans to complete an evaluation of the project by June. For Congress, the Office of Technology Assessment is also evaluating the genome project. A draft of that report may be available this summer, although it is not officially due until the end of the year.

Joseph Palca



Industry (MITI) and the Ministry of Post and Telecommunications (MPT) in October 1985 (*Nature* 317, 99; 1985).

Ever since Japan Key Tech came into existence, the two ministries have been vying for the most prestigious project to be funded by it. This month MPT will establish an institute for the development of next-generation space communications, such as large-diameter antennas and miniaturized transmitters for space stations, with funding of ¥6,000 million over eight years. But this pales in comparison with MITI's plans to build a protein engineering research institute.

About two years ago MITI counselled a

Investigations into NIH fraud allegations end with suicide

Washington

AN investigation into possible scientific fraud at the National Institutes of Health (NIH) has had tragic consequences. Apparently as a result of emotional pressure caused by an inquiry that focused on a researcher's conduct in his former laboratory, Howard Eisen committed suicide on 7 February, 10 days after appearing before a panel looking into the affair.

Eisen was in the Laboratory of Developmental Pharmacology at the National Institute of Child Health and Human Development (NICHD), and chief of the section on regulation of gene expression. The NIH inquiry focuses on a paper entitled "Analysis of mammalian P450 catalytic activities by genetic engineering and expression in yeast." According to Eisen's widow Laura, a research associate in chemistry at the Uniformed Services University of the Health Sciences, most of the data for the paper were gathered by Alok Bandyopadhyay. Laura Eisen says Bandyopadhyay was hired last summer by Daniel Nebert — head of Howard Eisen's section — to work in Eisen's laboratory. According to Laura Eisen, Nebert wrote the paper, although Bandyopadhyay was first author. Howard Eisen reviewed the paper, and he was one of the authors, she says.

The paper was intended for publication in the Proceedings of the National Academy of Sciences, but certain inconsistencies in the data were detected before it was actually submitted for publication. Those discrepancies prompted a careful review

of Bandyopadhyay's work at NIH. Laura Eisen says her husband performed experiments that convinced him that Bandyopadhyay had adulterated his data. She says not only was there evidence that there were serious problems with Bandyopadhyay's research, but close scrutiny subsequently turned up numerous inconsistencies in his curriculum vitae. Unfortunately, Bandyopadhyay could not be reached for comment.

Results of this preliminary scrutiny prompted the NICHD to terminate Bandyopadhyay's employment last November. NICHD director Duane Alexander says that the NIH follows specific procedures in any case of suspected fraud, both within and without NIH. So on 8 January this year deputy director Joseph E. Rall named a three member panel with the object of looking into "serious discrepancies in the paper".

The panel comprises Maxine Singer of the National Cancer Institute and two other members, one from NIH and one from outside the institutes who acts as chair. The panel held its first meeting on 28 January, when both Howard Eisen and Bandyopadhyay were expected to be interviewed. But Bandyopadhyay never arrived, and after waiting several hours the meeting broke up with no substantive ground being covered.

Howard Eisen was extremely upset by his meeting with the panel according to his widow. She says he insisted on going back to his laboratory to make certain that his checks of Bandyopadhyay's work had

AAAS reviewing the situation

Chicago

THE American Association for the Advancement of Science (AAAS) boasted of a remarkable coup at its annual meeting here this week — the appointment of Dr Alan Trivelpiece as its chief executive officer to succeed Mr William Carey, who is retiring. Dr Trivelpiece has been for several years responsible at the Department of Energy for, among other things, US policy on high-energy physics. He is believed to be the chief architect of the US government's decision, announced two weeks ago, to back the project to build the Superconducting Super Collider (see page 654).

In contrast with previous years, the AAAS this year has no fierce complaint to make of the federal budget for the year ahead. Neither the present nor the putative executive officer could do other than applaud the hope that Congress will allow the doubling of the budget of the National Science Foundation (NSF) over the next

five years, one of the outstanding ingredients of the administration's budget for the financial year beginning next October. But Mr Gerard Piel, chairman of the AAAS board of directors, "deplored" the NSF's recent practice of setting up university-based research centres whose effect, he said, is to undermine the autonomy of the host universities.

The AAAS itself seems also to be going through a period of heart-searching about its future. A downbeat statement in the association's house journal, *Science*, which records the circumstances leading to the demise of its monthly offshoot *Science 86*, also says that the future of the annual meeting is being re-assessed because of poor attendances. Unsystematic sampling of this week's sessions reveals that distinguished speakers are often as numerous as those who have come to listen. But hotels have apparently been booked for the next two years. John Maddox

Two British scientists share Wolf Prize

Rehovot £8

Two noted British biochemists will share the \$100,000 Wolf Prize in Chemistry for 1987. They are Sir David Phillips of the University of Oxford and Professor David M. Blow of Imperial College, London, who are being honoured "for their contributions to protein X-ray crystallography and to the elucidation of structures of enzymes and their mechanism of action".

Phillips and Blow will receive the Wolf Prize from the president of Israel, Dr



Sir David Phillips (left) and David Blow

Chaim Herzog, in ceremonies at the Knesset in Jerusalem on 31 May, when awards will also be presented for achievements in chemistry, mathematics, medicine, agriculture, physics and the arts.

Nechemia Meyers

been accurately prepared. Laura Eisen says her husband was not thinking rationally following the meeting. Upset from the start by the apparent fraud, Laura Eisen says "something snapped" after the 28 January meeting.

Friends and associates of Howard Eisen say the idea that he could have been involved in anything fraudulent is unthinkable. Jeffrey Harmon, a colleague of Laura Eisen at the Uniformed Services University of the Health Sciences who had collaborated with Howard Eisen in the past, says "there's no way Howie could have been involved in any kind of cheating".

Although he had minimal contact with Eisen, Duane Alexander describes him as "as excellent scientist". NIH has made no allegations against Eisen.

Howard Eisen came to NIH in 1971. He was graduated from Harvard College in 1964 and Harvard Medical School in 1969. Eisen did a two-year residence at Boston City Hospital before joining NIH. His work has been primarily on glucocorticoid receptors.

NIH is still planning to continue its investigation into the fraud allegations.

Joseph Palca

Call for replacement for UGC and bigger role for industry

London

BRITISH industrialists and women must play a greater part in influencing the education and research needs of the nation while government, via a special commission, should have immediately available expert counsel on education.

These recommendations have emerged from a committee created to review the University Grants Committee (UGC) — the principal financial watchdog of higher education. The committee, chaired by Lord Croham, chairman of the bankers Guinness Peat and former head of the Home Civil Service, concludes that "national needs" are being ignored by the educational establishment and that a more "open" approach is needed to ensure that those demands are satisfied.

The UGC should be replaced by a council with no more than 15 members, says the committee's report, while a newly created UK Education Commission would provide advice on the educational needs of industry and commerce.

The new bodies would have a membership that reflects national interests. The committee was emphatic that industry and commerce "Do not at present have a satisfactory focus for the expression of their views and on that account were not playing as full a part as they might in national debate on education".

The UGC, established in 1919, currently has 17 members but more than a dozen sub-committees. The proposed council would be steered principally through the new office of a director general, a full-time member who would preside in the chairman's absence. The profile of that chairman must also change. He or she must be "An eminent person from outside the academic world".

The constitution of the council would be changed dramatically with the membership no longer dominated by prominent academics. The UGC, presently chaired by Sir Peter Swinnerton-Dyer, has only one woman member — a fact that is singled out for criticism by Croham. A council dominated by men would be unlikely to explore how women could play a fuller role in universities, says the report.

While not overtly criticizing the present system, the nine members of the Croham committee conclude that "Through no fault of its present members, the UGC lacks the necessary balance of experience". The Croham report emphasized that the academic judgement frequently asked of the UGC should be conducted within the perspective of "public interest". There must be a wider economic and social perspective when addressing these questions.

The UK Education Committee which could either be assembled periodically or be in permanent session, would be responsible for advising the government "on national goals for the education service as a whole, and on any reforms which might be necessary". Both new bodies are to be assisted, if the recommendations gain approval, by triennial budgets established for the universities, assuming the annual inflation rate remains below five per cent.

The coordination of the management and funding of medical education also needs attention, says the Croham study. A sub-group led by Lord Butterworth, former vice-chancellor of Warwick University, specifically investigated the problems. The current machinery which depends on joint policies hatched by the Department of Education and Science, the Department of Health and Social Security, and the UGC is weak, says Croham, "with the result that too much is left to chance. That is a matter which needs urgently to be tackled by the departments concerned".

Bill Johnstone

The Croham report is published at £7.20 as "Review of the University Grants Committee", Command 81, HMSO.

New technique to gauge sea ice

London

A NEW method, employing the characteristics of impulse radar, has been developed to measure the thickness of sea ice, allowing oil companies to determine easily the forces exerted by icefloes against structures such as oil or gas platforms.

The radar unit, developed jointly by Exxon Production Research Company and the British-based Cambridge Consultants, is mounted on a helicopter and emits short pulses of electromagnetic energy from antennas fitted on one side of the aircraft. The energy is in part reflected from the top of the ice and also from the interface between the bottom of the ice and the sea water. These reflections are collated by a receiving antenna housed at the other side of the helicopter.

The time difference between the received signals and the velocity of this radar energy as it travels in the ice allows the thickness to be easily determined. The device, which can accurately measure up to 30 ft to within one foot, supersedes manual drilling techniques. According to the inventors of the impulse radar device, "it is possible to collect more data in 30 minutes than can be obtained by a six-man crew drilling for two days".

Bill Johnstone

All change at the top for India's nuclear agency

New Delhi

†35

THE crisis in India's Department of Atomic Energy (DAE; see *Nature* 325, 565; 1987) has deepened with the resignation of Dr P.K. Iyengar, director of the Bhabha Atomic Research Centre, the physicist credited with the design of the plutonium device that India exploded in 1974. His departure follows the announcement on 9 February of the appointment of his rival, Dr M.R. Srinivasan, head of the Nuclear Power Board, as successor to Dr Raja Ramanna, the retiring chairman of the Atomic Energy Commission (AEC).

Iyengar, who is 55, said he was seeking voluntary retirement after 32 years of service. Dr N. Srinivasan, a close associate of Iyengar, has also resigned from his post as director of heavy-water projects but for reasons, he says, unconnected with the controversy over who should be the new chairman of AEC.

The Prime Minister, Mr Rajiv Gandhi, who is also in charge of atomic energy, is upset over the row among senior scientists at a time when the threat of a Pakistani nuclear device is looming large and when India is launching an ambitious 15-year programme to expand nuclear power capacity from 1,000 MW at present to 10,000 MW. In the wake of criticism in the media about the inept handling of the succession at AEC, Mr Gandhi personally intervened, but was unable to dissuade Iyengar from resigning.

The vertical structure of Indian scientific organizations and the immense power bestowed on their heads leads to intense competition for senior posts. The AEC chairman automatically becomes secretary to the government; he also draws the highest salary of any scientist.

M.R. Srinivasan, who will take over from Ramanna at AEC on 1 March, is a British-trained mechanical engineer. He joined DAE in 1955 and was in charge of its nuclear power projects division until, in 1984, this was reconstituted as the Nuclear Power Board, whose chairman he became. No successors to Iyengar and Srinivasan have yet been named.

In the meantime, Mr Gandhi has set up a committee under his science adviser, Professor M.G.K. Menon, to study the Soviet offer of two 440-MW light-water reactors. When the offer was made four years ago it was rejected by Ramanna and Iyengar because it did not fit the Indian nuclear programme. Following so soon after Mr Mikhail Gorbachev's visit to India, the prime minister's action is interpreted as a sign that India may now import the Soviet reactors.

K. S. Jayaraman

More compensation in Finland for nuclear accident victims £57

London

FINLAND'S Ministry of Trade and Industry is preparing an amendment to the law on nuclear accidents that will considerably increase the maximum compensation payable to victims. According to the Ministry of Justice, the maximum liability of nuclear power companies will probably be increased tenfold to Fmk 500 million (about £70 million) per station, and the maximum state liability by a factor of 2.5 to FM 1000 million. The bill has already been passed by the present parliament, but, having been introduced late in the parliament's four-year life, it will have to be resubmitted to the incoming parliament after the March general election. In the aftermath of Chernobyl, however, there seems little doubt that the bill will become law.

Although Finland escaped the worst of the first wave of Chernobyl fallout, the government's handling of the incident has been widely criticized. In Finland, the task of informing the public was left to the nuclear 'experts', in particular the head of the Institute of Radiation Protection, Antti Vuorinen, who said there was no information he could give except that radiation levels were up — but nowhere near the danger level. In fact, the radiation levels came close to the danger level only at the military base of Kajaani but the experts' attitude did not inspire public confidence. Doctors in particular complained that they had to try to cull information from the general press, which had frequently either misunderstood the little told them, or were reprinting foreign speculations.

Later studies of information flow and media coverage, made at the Universities of Helsinki and Tampere, compared the government's handling of the emergency unfavourably to the more open approach followed in neighbouring Sweden, though the Tampere study established that some nuclear experts felt it was unfair to leave the reporting of nuclear power issues in the hands of editors, most of whom openly admit to being opposed to nuclear power.

But despite this, the effect of Chernobyl on Finnish public opinion was less marked than might have been expected, the Tampere study concluded. Public opinion polls gave a level of 60 per cent opposed to nuclear power in May, compared with 33 per cent before Chernobyl. But this had fallen to 58 per cent by June and to 44 per cent by December. The time-scale, however, was significant — the accident came just as the government was about to give the go-ahead for a fifth nuclear power station in Finland, a scheme that has now been shelved.

Equally, however, post-Chernobyl de-

mands that Finland should phase out the existing stations were summarily rejected. Apart from peat and 'energy woods', Finland has no domestic energy resources, and imported fossil fuels, including Soviet oil, are not only expensive but are also harmful to the country's main natural asset, the forests.

From the beginning of their nuclear power programme, the Finns have been mindful of the dangers. Two of their four existing nuclear stations were purchased from Sweden and two from the Soviet Union. The latter are of VVER-type — the RBMK (the type used at Chernobyl) has so far never been installed outside the Soviet Union. The Finnish VVERs, however, have a feature not found in the

Soviet prototype — a Westinghouse containment building (Finnish wits call the composite an 'Eastinghouse').

But, since Chernobyl, the main nuclear power threat has seemed, to many Finns, not their own 'Eastinghouses', but the other Soviet RBMK stations, at Ignalina and Leningrad, far nearer than Chernobyl. Finland has, of course, acceded to the new Convention on the Rapid Notification of nuclear accidents, drawn up by the International Atomic Energy Agency last October, and has also (two weeks ago) concluded a bilateral agreement on nuclear safety with the Soviet Union.

One idea now current in Finland is that this cooperation could include the construction by the Finns of containment vessels for the Ignalina and Leningrad RBMKs. The Soviet view is that containment buildings are unnecessary and could not have prevented the escape of radioactive material at Chernobyl. **Vera Rich**

Texas amongst early contenders in the race to subsidize the SSC

Washington

THE Department of Energy (DoE) has sounded the starting gun and the race to play host to the Superconducting Super Collider (SSC) is under way. On 10 February, Secretary of Energy John Herrington explained how and when a site for the SSC, which will be the largest particle accelerator ever built, will be selected. The prize for the state that wins will be construction jobs and a long-term increase in local spending.

Construction of the \$4,400 million instrument is due to begin in 1989 and to be completed in seven years. Offers of sites will first be reviewed by a panel whose members will be selected by the National

state will have offered to provide the land on which the collider is to be built, but the bid will probably have to contain much more than that. One candidate site is that of Fermilab in Illinois, where the existing proton accelerator could serve as an injector for the new machine, perhaps saving a few hundred million dollars. At least two other states are said to have made offers to match that saving with cash.

Texas has been interested since the project was first discussed by the High-Energy Physics Advisory Panel early in the decade, while New York and California are among the others now interested. Overseas contributions may also help. Herrington said that he "wouldn't be surprised" if foreign contributions accounted for 25–50 per cent of the construction cost.

While the future of the SSC will rest ultimately with Congress, the DoE has not asked for new funds for the next fiscal year, beginning on 1 October, but assesses that the \$35 million needed will be reallocated from existing funds. But winning congressional approval for the projected \$348 million expenditure for the collider in fiscal year 1989 "will be a battle," according to one Senate staff member. The balance of the costs will have to be agreed by the next Congress.

The operating costs of the SSC are expected to be \$270 million a year, and will be borne by the DoE. Herrington said that the funds would not be diverted from other programmes such as those at Fermilab and the Stanford Linear Accelerator Center, but a spokesman added later that those laboratories might not be operating when the SSC is on line.

Steven Dickman



Academy of Sciences and the National Academy of Engineering. On the basis of the panel's recommendations, the DoE will select the best site by July next year, subject to later environmental and geological investigation.

One of the considerations will be the guarantees given by bidders to help contain the cost. Herrington said last week that the DoE is assuming that the chosen

Japan looks to sea for the extra acres and airports

Tokyo

JAPAN is expanding into the sea. Several multi-billion dollar projects to construct reclaimed islands off Japan's coast are on the drawing board and reclamation work for the new Kansai International Airport is already under way. This is a massive operation calling for the creation of a man-made island of more than 5 km² in Osaka Bay and connected to the mainland by a 4-km road and rail bridge. The airport is due to be completed in 1993 at a cost of ¥1 million million (\$6,600 million); a further ¥2.5 million million will be spent on construction of railways, expressways, a residential area, parks, a water supply and a sewage system. Future plans call for the airport to be more than doubled in size to accommodate two additional runways.

The soft clay floor of the bay will pose problems for Japan's civil engineers. After ground improvement by sand compaction and drainage techniques, an 11-km wall will be built around the site before infilling with about 200 million m³ of sand and soil derived from the mainland.

This is not the first or last man-made island of such a scale to be built by Japan. Port Island, of comparable size to the new airport, was completed off Kobe in 1981, and Rokko Island, its twin nearby, is nearing completion. Osaka plans to create two new islands in its harbour by dumping domestic and industrial garbage behind retaining walls. And Tokyo has the most ambitious plans of all — the National Land Agency has called for the reclamation of 17 km² along the waterfront of Tokyo Bay by 1995, and under a cooperative government-private venture a bridge and tunnel will span the 15 km of Tokyo Bay between Kawasaki and Chiba Prefecture via a couple of artificial islands. The price of mainland property has made this reclamation programme attractive.

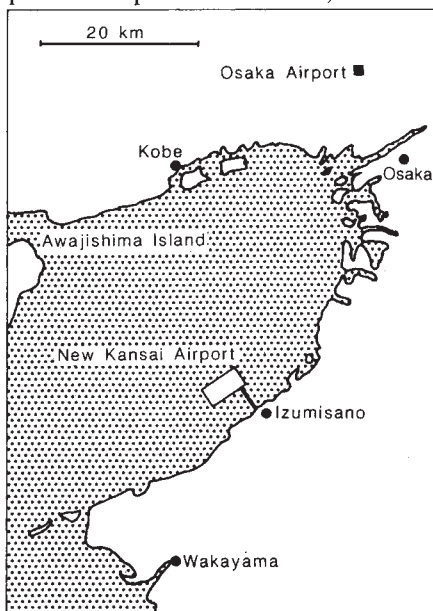
When the national government recently asked Tokyo metropolitan government to construct a loop to link the city centre with the coastal redevelopment plan, officials were appalled to discover that a 1.35-km section between Toranomon and Shimabashi, although costing only ¥2,000 million to build, would entail spending ¥430,000 million to cover land purchase for the 40-km wide road, the equivalent of more than \$2,000 million per kilometre of highway.

Redevelopment itself only tends to worsen the situation: when Tokyo announced plans to move the metropolitan government offices from the centre of town to a new site in Shinjuku on the outskirts, land prices in urban areas within 30 minutes' commuting distance of Shinjuku doubled in a few years to well over a

million yen (\$6,600) per m².

Land prices are not the only problem. Many old city areas lack the necessary infrastructure, roads and sewage systems, for redevelopment; Osaka may boast a bullet train, but it still has open sewers that overflow during the rainy season.

So offshore development has proved attractive. There new land can be created for as little as ¥100,000 per m². The only objections come from local fishermen, who can usually be bought off with suitable compensation — for the new Kansai airport more than ¥40,000 million was paid in compensation to the 3,400 fisher-



man of Hyogo and Osaka Prefectures. Few of these fishermen ever fish anywhere near the site, but in Japan a blanket approach to compensation is favoured to quell dissent among those most affected.

The new reclamation projects are joint government-industry ventures set up under a new law introduced last year to encourage private investment in public works. Main contractors are chosen under a designated bidder system, which means only select companies can bid for the project. Fifty per cent of the funding for the project is provided by the companies themselves, which buy low interest bonds from the local government.

The prospects for any foreign companies with plans to compete with local constructors seem slim. But following intense diplomatic efforts, the United States has managed to pick up at least one crumb from the Kansai airport contract. Bechtel Civil Corporation, a San Francisco-based engineering consulting firm, has won a ¥30 million subcontract to survey facilities at international airports overseas.

David Swinbanks

Export licence rules to ease?

Washington

IN response to the publication of a National Academy of Science report critical of US export control policy, the Department of Commerce is proposing significant changes in its export licensing procedures. The aim is to stop "the growing shift away from American products" without jeopardizing national security.

Secretary of Commerce Malcolm Baldrige announced the proposed changes last week. Although some need further approval, either from the White House or from Congress, others will go into effect immediately. This week, for example, the department is expected to publish in the *Federal Register* its revised plans for the general certified end user (G-CEU) licence. Dubbed the "gold card" scheme when it was first suggested last summer (see *Nature* 323, 100; 1986), it provoked strong criticism from industry and has been substantially revised as a result. Now, G-CEUs will be awarded to companies, such as Fiat in Italy, controlled by CoCom (Coordinating Committee on Export Controls) governments. A second general licence would allow routine shipments to all agencies of CoCom governments. Future efforts will extend G-CEUs to other Western companies.

Another change expected to be welcomed by industry will be a loosening of the so-called parts and components regulations. At present, anything manufactured outside the United States containing controlled US parts still requires a US export licence if re-exported. The new regulations will require licences for products containing some fraction (rumoured to be 20 per cent) of US parts or components.

Boyd McKelvin, head of the Industry Coalition on Technology Transfer, says the Commerce Department's plans are a "tremendous step in the right direction". He says the plans to eliminate many controls on low technology items could have a large impact on trade. McKelvin says the large US trade deficit coupled with the National Academy of Science's report allowed industry's voice to be heard.

But the Department of Defense has not been overwhelmingly enthusiastic about the Commerce Department's proposals, even though the G-CEU scheme came originally from Deputy Undersecretary of Defense, Stephen Bryen. There is still concern that tough controls are needed to prevent the Soviet Union from gaining access to sensitive US technology. The Defense Department is preparing its own agenda for revising export controls, said a Pentagon spokesman. Ultimately, the National Security Council will have to balance the concerns.

Joseph Palca

Accuracy, statistics and fraud

SIR—The catalogue of errors in scientific publications exemplified in the article by Stewart and Feder¹ will not surprise any statistician or biometrician, least of all one who has acted as a statistical referee of biological papers submitted for publication. Stewart and Feder refer to a paper², where “almost all the data ... are contained in three figures which cannot be reconciled with each other”, and this experience could be mirrored only too often.

Biometricians, and the Biometric Society, have been concerned with this problem for many years, an early example being the critical review by Mead and Pike³. The British region of the society held a full-day meeting in December 1981 on “Statistical Presentation of Information in Biological Journals”, where journal editors and statistical consultants put forward views that were discussed by a panel of experts and the audience. The *British Medical Journal* and the *Journal of Ecology*, representing very different areas of biology, were subjected to especial scrutiny. Although we believe the situation to have improved, there is still a long way to go. That the problem persists is shown by a letter⁴ that you published in the same week as the article by Stewart and Feder, where a biometrician criticized a paper for a simple arithmetical error.

The problem is not that there may be different possible statistical interpretations of the same data. Certainly there are different statistical techniques available for analysing the same data, and indeed there is a proliferation of computer packages that vary considerably in their statistical reliability. A statistical technique that I would not advocate could appear in a paper submitted to me as a referee: I might be slightly unhappy but, provided the interpretation was correct, I should not recommend rejection of the paper. Too often, however, as in the example quoted by Stewart and Feder, incorrect statistics mean that a paper is actually wrong. There is no need to postulate outright fraud. Sometimes authors genuinely do not understand their results — and this usually means that they have either not sought statistical advice or not made proper use of it. At other times, research workers will do an experiment to test a hypothesis and then, consciously or unconsciously, ignore results that do not conform to their preconceptions. With the pressure to publish, there is a tendency to cut corners.

It is wrong to blame only authors for making misleading statements¹. Too many referees are insufficiently critical, and in-

deed too many editors do not seem aware of their responsibilities. The position might well be improved if you, sir, and your fellow-editors used referees who are able to distinguish minor weaknesses from substantial errors caused by inability to use correct statistical methods. The consequence might be fewer papers published, but those accepted would be of higher quality.

G. H. FREEMAN

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1. Stewart, W. W. & Feder, N. *Nature* **325**, 207–212 (1987).
2. *Ann. Surg.* **194**, 189–192 (1981).
3. *Biometrics* **31**, 803–851 (1975).
4. Perry, J. *Nature* **325**, 202 (1987).

SIR—There is a serious methodological error in the paper by Stewart and Feder (*Nature* **325**, 207; 1987), for there is no control group. Their own publications, submitted to independent assessors, could have fulfilled this role.

A. M. W. PORTER

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SIR—Two recent papers have significant bearing on an important issue discussed in *Nature* recently (**325**, 207; 1987), namely the integrity of the scientific literature and specifically the problem of “scientific fraud”.

In the first of these papers¹, de Lacey and his colleagues asked the question, “How accurate are quotations and references in medical journals?” Their depressing answer was — not very! “...the original author was misquoted in 15% of all references, and most of the errors would have misled readers. Errors in citation occurred in 24%, of which 8% were major errors — that is, they prevented immediate identification of the source of the reference.”

In the second paper², I tried to assess the occurrence and the significance of ‘self-confessed’ errors in the biological literature, that is those instances where authors subsequently admit that something they had earlier published had not actually happened the way they originally said it had.

Again, the result of this survey was depressing. In a number of biological journals, including acknowledged leading journals, some 2 per cent of all papers are subsequently modified by their authors. While many of these modifications are primarily cosmetic, perhaps half of them are highly significant — ‘we didn’t actually use the buffer, drug, concentration, machines settings, number of animals, ...’ we originally said we did.

The number of such admissions may seem to be small, but how many such mis-

takes, all of which render impossible the repetition of the work as originally published, are not detected ... or not admitted ... or not published?

At question is a fundamental point, the ‘accuracy’ of the published paper; all of us — readers, reviewers, editors — have to believe that what was reported did in fact take place.

Fraud, that is, a deliberate intention to mislead, which is (it is to be hoped) only rare, will account for some inaccuracies. But it also seems that a lack of sufficient care may be widespread and is responsible for many more inaccuracies. We readily and rightly condemn the fraudulent, but the end result from the activity of the careless is equally damaging to the framework, the reputation and the progress of science. Should not the careless be equally condemned, and carelessness guarded against with a vigour equal to that with which we guard against fraud?

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1. de Lacey, G., Record, C. & Wade J. *Br. med. J.* **291**, 884–886 (1985).
2. Sabine, J. R. *Bioscience* **35**, 358–363 (1985).

Dissent in Singapore

SIR—I should like to point out some errors in the letter “Dissent disallowed in Singapore” by Louis Mahadevan (*Nature* **324** 298; 1986).

Firstly Mr J. B. Jeyaretnam was gaoled not for being “the voice of dissent in Singapore” but on a criminal offence. Jeyaretnam and Mr Wong Hong Toy were fined and gaoled for making a false declaration about their political party’s accounts. Both Jeyaretnam and Wong appealed to the Privy Council, which dismissed the appeal with costs. Is Mahadevan questioning the integrity and independence of the Privy Council?

Second, Mahadevan alleged that the voice of dissent in Singapore is to be silenced at any cost. Far from it. Jeyaretnam has continued to voice his dissent during and since his gaol term through local as well as foreign publications circulated in Singapore.

Third, Mahadevan alleged that two publications were sanctioned for reporting Jeyaretnam’s imprisonment and consequent loss of seat in Parliament. This is not true. The *Asian Wall Street Journal* was cited for contempt of the judiciary and apologized in court. *Time* magazine was sanctioned because it refused to publish corrections of errors in its article; it eventually admitted the errors and printed the corrections.

MARY SEET-CHENG

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Letters submitted for Correspondence should be typed, double-spaced, on one side of the paper only. □

Order for order-disorder?

There seems to be a simple rule for telling when some semiconducting ternary alloys have an order-disorder transition below the melting point. Will it apply to potentially commercial semiconductors?

CHEMISTRY at its most appealing is applied common sense. This is the spirit in which people in the nineteenth century were able to devise empirically based rules relating properties of one kind to those of another for some class of chemicals. Sometimes the outcome would be simply a useful way of codifying data and of making plausible predictions, as in the empirical statement that the entropy of vaporization at the boiling point is roughly constant, called Trouton's rule. But in the early decades of the century, much bigger prizes had been won from these empirical relationships. Would there have been an atomic theory in Dalton's time without the law of multiple proportions, for example?

So it is good that the tradition still flourishes, even in unlikely connections. Thus Alex Zunger, from the Solar Energy Research Institute at Golden, Colorado, has just applied exactly such a technique to the codification of order-disorder transitions in the three-component alloys on which those who design new semiconductors have set their hearts (*Appl. Phys. Lett.* **50**, 164; 1987).

The importance of the problem is evident. Sooner or later, no doubt, silicon will be replaced by materials such as gallium arsenide (GaAs) or indium antimonide (InSb) in the microcircuits on which we all rely. The principle, which explains why chemists are closely engaged with these developments, is simply that if, say, germanium is a semiconductor, an alloy of the two elements on either side of it in the Periodic Table, Ga and As, will almost certainly be a semiconductor as well. Experiment indeed confirms that the outer-shell electrons of Group III gallium and Group V arsenic fill the valence band of an equi-atomic alloy, but leave the conduction band essentially empty.

For the past decade, chemists have been busily ringing the changes on this and other recipes for making semiconductors, knowing how great the practical benefits could be. GaAs should be faster than silicon, while sandwiching an alloy in which 30 per cent of the Ga atoms are replaced by Al (also in Group III) between slices of GaAs will make an infrared laser as well. The practical snag is the difficulty of controlling the stoichiometric balance of the composition to a few parts in 100 million, the level at which electronic doping by foreign ingredients can be significant.

Zunger is concerned with a lesser but still important snag — the circumstance

that semiconductors that are ternary compounds should in principle exist in one or other of two alternative states, ordered and disordered. In the case of $\text{Al}_x\text{Ga}_{1-x}\text{As}$, for example, where Al and Ga are electronic substitutes for each other and where the electronegativity of the As is likely to give it anionic properties and thus large size (relative to the Al and Ga atoms in the lattice), it is an interesting but open question whether the Al and Ga atoms are distributed randomly and, if not, whether they are ordered in clusters or in some other way. Moreover, the electronic properties of the material could depend on what the answer is. The transition from order to disorder should decrease the band-gap, for example.

Luckily, most semiconductors appear to be built on the architecture of the diamond lattice — that of a tetrahedron in which an anionic atom is surrounded by four cationic atoms (and, for that matter, vice versa), which allows Zunger plausibly to compare semiconductors whose order-disorder transitions are likely to occur at low temperatures where they cannot easily be observed with those of other materials where the transition has actually been observed. Specifically, he considers the range of ternary alloys or molecular compounds in which two electronegative Group V or VI atoms are combined respectively with one Group I and one Group IV atom or with one Group II atom and one from Group V. AgInTe , might stand for the first class of compounds ZnSIAs , for the second. The materials are respectively chalcopyrites and pnictides. Zunger lists more than 30 known semiconductors of both kinds.

These materials differ from those at the centre of the device-makers' attention in that the cationic components, not being isovalent, are far from being electronic substitutes for each other, as Ga and Al are. Zunger points to two energetic consequences of this state of affairs for the order-disorder problem.

First, the ordered state in which each anionic atom (from group V or group VI) is surrounded tetrahedrally by two pairs of cationic atoms is likely to persist to much higher temperatures than in alloys in which, say, Ga and Al atoms may substitute for each other with negligible energetic penalty. This is what the experiments show: order-disorder transitions have been observed in 13 out of the 30-odd chalcopyrite semiconductors at tempera-

tures not far short of the melting point (about 1,000 °C.) The snag, for materials such as $\text{Al}_x\text{Ga}_{1-x}\text{As}$, is that the energetic differences between the cationic components are so small that the transition, if it occurs, will be at too low a temperature for it to be practicable to tell whether the usually disordered state of the alloy is the lowest or merely a metastable state.

Zunger's second observation is where the common sense comes in. If there are low-temperature ordered states in ternary alloys of this kind, they will not be tetrahedrally symmetric about the cations, but distorted so as best to accommodate the two different ionic radii of the two pairs of cations. The disordered state, on the other hand, in which cations are distributed at random about their central anions, must be tetrahedrally symmetric, which means that the lattice embodies a substantial penalty in internal energy (or in enthalpy) in exchange for the benefits of the extra entropy that comes from the disorder.

What Zunger does is what chemists have been doing for two centuries. He defines a parameter (called u) to represent the measured departure from tetrahedral symmetry in the ordered phases of the chalcopyrite alloys. (Numerically, u is determined by the difference, in the ordered phase, between the distances from the central anion to each of the two different kinds of cations.) Intuitively, one would expect that if there are ternary alloys that make a transition from order to disorder before they melt, they will be the alloys for which the value of u is the smaller.

This, magically, is how it turns out. For the 23 compounds for which data exist, the 13 that make a transition to disorder before melting have u less than 0.265; the remaining ten, which remain ordered up to the melting point, have larger values of u . Emboldened by the fact that there are no known exceptions to his rule, Zunger goes on to predict which side of the fence ten compounds not yet made will fall.

There are several lessons to be learned from this elegant exercise. The argument that lattice strain may stabilize order may be extended to alloys of more immediate interest. It may even be possible to make a numerical connection between disorder and the reduction of the semiconductor band-gap it brings about. But the chief may be that there is mileage yet in a style of argument that seemed to go out of fashion when people took to chemistry by supercomputer.

John Maddox

Alzheimer's disease

Progress in molecular pathology

Brian H. Anderton *£52*

SENILE dementia of the Alzheimer type is believed to affect 5 per cent of people over 65 years of age. That the disease is a major health problem is reflected by the intense research activity aimed at elucidating the pathogenesis and cause of the disease. The techniques of molecular biology are now being brought to bear on this problem, and one of the first results is reported in a paper by Müller-Hill, Beyreuther, Masters and collaborators on page 733 of this issue¹.

Senile plaques and neurofibrillary tangles are characteristic of the histopathology of Alzheimer's brains. Two abnormal filamentous deposits are associated with these lesions and determination of the chemical composition of the filaments may provide clues about the underlying molecular pathology. The filaments that comprise the neurofibrillary tangles, paired helical filaments (PHFs), appear in the electron microscope to consist of two 10-nm wide filaments twisted about each other in pairs². PHFs are intracellular and, as well as being present as tangles in neuronal perikarya, are found in many of the abnormal dendritic and axonal processes, or neurites, that make up the periphery of the senile plaque. The senile plaque, an area of disorganized neuropil up to 150 µm across, often has an extracellular core which is a deposit of amyloid. This cerebral amyloid is ultrastructurally distinct from PHFs, consisting of 4–8-nm wide filaments that are not wound together in pairs³. Plaques and tangles can be widespread in several cortical areas, particularly in the hippocampus, of Alzheimer's brains and their numbers are related to the severity of dementia.

Cores

The cores of extracellular plaques have been isolated in several laboratories^{4–8}. There seem to be at least two components to the plaque core, an inorganic aluminosilicate⁹ and an abundant polypeptide of relative molecular mass (M_r) about 4,000, termed A4 and isolated previously by Masters *et al.*⁵. These authors obtained a partial amino-acid sequence for A4 and showed that it is probably identical or closely similar to the amyloid protein that Glenner and Wong had already isolated from cerebral blood vessels from patients with Alzheimer's disease and Down's syndrome and which the latter authors had also sequenced¹⁰. Immunohistochemical studies^{7,11,12} confirm that plaque-core A4 is related to vascular amyloid protein. The paper by Müller-Hill and his co-workers in this issue¹ reports the

isolation and sequence of a complementary DNA clone that seems to encode a much larger precursor protein of the amyloid A4 peptide and the authors speculate that A4 is derived from a membrane glycoprotein. They also report the complete amino-acid sequence of A4, which is either 42 or 43 residues long.

Müller-Hill's group screened a human fetal brain complementary DNA library with a synthetic oligonucleotide based on part of the A4 amino-acid sequence. The sequence of a 3,353-base pair apparent full-length DNA shows that the A4 sequence is within a much larger one encoding a protein of 695 amino-acid residues and of M_r about 79,000. The amino terminus of the long precursor begins with a signal sequence for translocation of the polypeptide through the endoplasmic reticulum and is followed by a cysteine-rich region up to residue 187, suggesting a disulphide-stabilized domain. Next there is a 100-residue segment rich in acidic side chains which the authors speculate may be involved in the electrical activity of cells. Two potential *N*-glycosylation sites run from amino-acid residues 467 to 469 and 496 to 498 and a hydrophobic sequence characteristic of a transmembrane domain runs from residues 625 to 648. The A4 sequence is between residues 597 and 638 and is therefore partially included in the putative transmembrane region. Overall, the A4 precursor has the characteristics of a membrane-spanning glycoprotein.

Alzheimer's brains and normal adult human cortex contain two transcripts of 3.2 and 3.4 kilobases, respectively, detected by Müller-Hill and co-workers¹ using their clone. Thus, the gene encoding the A4 sequence is not uniquely expressed in Alzheimer's disease. Gajdusek reported recently (*4th meeting of the International Study Group on the Pharmacology of Memory Disorders Associated with Aging* Zurich, 16–18 January 1987) that his group has independently isolated¹³ another human complementary DNA for the A4 precursor and shows that the gene is expressed in thymus, muscle and heart as well as brain but not in liver or placenta.

Gajdusek also reported that the gene is highly conserved because positive Southern blots have been obtained with genomic DNA from hamster, mouse, rat, chicken, lobster, *Drosophila* and yeast. The next stage will probably be to establish the tissue and subcellular distribution of the A4 precursor in healthy humans and in Alzheimer's disease and to try to determine how A4 is generated and deposited as amyloid in diseased brain.

Müller-Hill's group shows¹ and Gajdusek confirms¹³ that the A4 gene is located on human chromosome 21. This result is particularly interesting because trisomy of chromosome 21 results in Down's syndrome. Individuals with Down's syndrome develop a brain pathology indistinguishable from Alzheimer's disease by the time they become 60 years old. Because of this association with Down's syndrome, it has been thought for some time that the common form of Alzheimer's disease, which is not exclusively sporadic but often shows a familial tendency, might be linked to a gene on chromosome 21 that would cause Alzheimer's disease either by a gene-dosage effect (Down's syndrome) or as a result of a mutation (Alzheimer's disease). A much rarer form of Alzheimer's disease (familial Alzheimer's disease, FAD) is determined by a single autosomal dominant gene and, in carriers, leads to an earlier onset of dementia. St George-Hyslop reported in Zurich on restriction fragment length polymorphism analysis of four families with familial Alzheimer's disease. Using several markers for different regions of chromosome 21, he found that the FAD gene is possibly in the proximal region of the long arm of chromosome 21. This should not be taken as definitive as more pedigrees need to be investigated, and it is premature to assume that the A4 precursor gene is the Alzheimer's gene, although this is an intriguing possibility.

Relationship

What of the relationship of A4 to the PHF proteins in the tangles? Masters and Beyreuther have previously proposed¹⁴ that PHFs are also composed of A4 but that A4 is assembled in a different way from that in amyloid fibrils, possibly with some of the amino-terminal residues proteolytically removed; this model is largely based on their isolation and sequencing of A4 from fractions enriched in PHFs. However, other studies with antibodies against either PHFs or plaque and vascular amyloids fail to demonstrate cross-reactivity between the two filamentous proteins and do not confirm their hypothesis. One possible explanation for this discrepancy is that PHF preparations are contaminated by amyloid and, hence, the A4 in isolated PHFs does not originate from the filaments but is from amyloid⁷. Beyreuther (*Dementia and Stroke. Biology of the Aging Brain* Heidelberg, 18–20 September 1986) and Gajdusek in Zurich both reported that A4 is extracted from PHF fractions isolated from brain tissue of cases of the Guam Parkinson-dementia complex. Because neurofibrillary tangles without plaques are the hallmark of this disease, these authors argue that A4 must be a genuine component of PHFs and does not come from contaminating amyloid.

Other investigations^{16–23} have shown

that certain antibodies against neurofilaments and against microtubule-associated proteins, particularly tau, label PHFs. Microtubule-associated tau, and possibly the neurofilaments that are incorporated into PHFs, may be abnormally phosphorylated. It has been reported²⁴ that tau protein isolated from Alzheimer's brains is defective in promoting microtubule assembly because it is abnormally phosphorylated. Davies and Wlozozin reported (16th American Society for Neuroscience meeting, Washington DC, November) that a polypeptide of M_r 68,000 that is expressed at elevated levels in many neurons in Alzheimer's disease²⁵ may be a protein kinase.

But there are no substantiated reports that the structural protein of microtubules, tubulin itself, is found in PHFs and the neurofilament proteins in PHFs seem to be derived proteolytically from the side-arms that crosslink neurofilaments to one another and to other organelles. Thus, whether or not A4 is complexed with the cytoskeletal components of PHFs, it will be important to see if the post-translational changes that produce A4 from its precursor, possibly located in the plasma membrane, are similar to those that affect the cytoskeleton. The obvious candidates at the moment for the agents bringing about these changes are protein kinases and proteases. □

- Kang, J. et al. *Nature* **325**, 733-737 (1987).
- Wisniewski, C.M., Crowther, R.A., Stewart, M. & Roth, M. *J. Cell Biol.* **100**, 1905-1912 (1985).
- Merz, P.A. et al. *Acta neuropath.* **60**, 113-124 (1983).
- Allsop, D., Landon, M. & Kidd, M. *Brain Res.* **259**, 348-352 (1983).
- Masters, C.L. et al. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4245-4249 (1985).
- Roher, A., Wolfe, D., Palutke, M. & KuKuruga, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2662-2666 (1986).
- Selkoe, D.J., Abraham, C.R., Podlinsky, M.B. & Duffy, L.K. *J. Neurochem.* **46**, 1820-1834 (1986).
- Gorevic, P.D. et al. *J. Neuropath. exp. Neurol.* **45**, 647-664 (1986).
- Candy, J.M. et al. *Lancet* **i**, 354-357 (1986).
- Glennner, G.G. & Wong, C.W. *Biochem. biophys. Res. Commun.* **120**, 885-890; **122**, 1131-1135 (1984).
- Wong, C.W., Quaranta, V. & Glennner, G.G. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8729-8732 (1985).
- Allsop, D. et al. *Neurosci. Lett.* **68**, 252-256 (1986).
- Goldgaber, D., Lerman, M.I., McBride, W.O., Sallow, V. & Gajdusek, D.C. *Science* (in the press).
- Masters, C.L. et al. *EMBO J.* **4**, 2757-2763 (1985).
- Anderton, B.H. & Miller, C. *Trends Neurosci.* **9**, 337-338 (1986).
- Brion, J.-P., van den Bosch, Ph., de Aguilar, P. & Flament-Durand, J. in *Advances in Applied Neurological Sciences. Senile Dementia of the Alzheimer Type* (eds Traber, J. & Gispén, W.H.) 164-174 (Springer, Berlin, 1985).
- Kosik, K.S., Joachim, C.L. & Selkoe, D.J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4044-4048 (1986).
- Wood, J.G., Mirra, S.S., Pollock, N.J. & Binder, L.I. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4040-4043 (1986).
- Grundke-Iqbal, I. et al. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4913-4917 (1986).
- Perry, G., Rizzuto, N., Autilio-Gambetti, L. & Gambetti, P. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3916-3920 (1985).
- Miller, C.C.J. et al. *EMBO J.* **5**, 269-276 (1986).
- Sternberger, N.H., Sternberger, L.A. & Ulrich, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4274-4276 (1985).
- Haugh, M.C., Probst, A., Ulrich, J., Kahn, J. & Anderton, B.H. *J. neurol. Neurosurg. Psychiat.* **49**, 1213-1220 (1986).
- Iqbal, K. et al. *Lancet* **ii**, 421-426 (1986).
- Wlozozin, B.L., Pruchnicki, A., Dickson, D.W. & Davies, P. *Science* **232**, 648-650 (1986).

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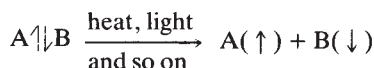
Physical chemistry

Long-lived superoxide radicals

Martyn C.R. Symons *£44*

ALTHOUGH radicals trapped in matrices have been studied for many years, trapping during the process of gel formation is described for the first time elsewhere in this issue by J. Ragai (*Nature* **325**, 703-705; 1987). The superoxide radical-anion, of major importance in the biochemistry of oxygen, is trapped by this procedure, and Ragai shows that it has characteristic electron spin resonance and infrared spectra that indicate a strong interaction between the anion and the gel. No doubt this interaction contributes considerably to the fact that these species remain trapped for long periods, even after heating to 110 °C.

To the chemist, the term 'radical' implies a molecular fragment containing an unpaired electron, which can often be viewed as a broken bond as implied in the following reaction



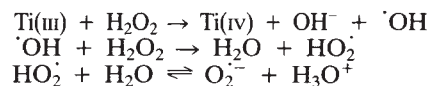
where $\downarrow \uparrow$ symbolizes two paired electrons. These radicals, normally written as $A^\cdot$ and $B^\cdot$, are often very reactive and can rarely be isolated. But they can be stabilized by trapping in solids, their reactivity being lost because of their immobility (Fig. 1). Only when the solid is heated can these radicals regain their mobility and hence react.

Ragai uses two techniques to establish the presence of radicals, infrared spectroscopy, which is non-specific, and the less well-known but extremely powerful technique of electron spin resonance spectroscopy, which is completely specific to species containing unpaired electrons. Hence, only radicals trapped in a matrix can produce an electron spin resonance spectrum — the matrix itself will be devoid of spectral features. Thus, electron spin resonance spectroscopy, which is extremely sensitive, can be used to detect

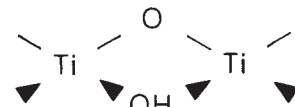
the 'fingerprint' radicals even in very low concentrations.

Radical-cations and radical-anions differ from neutral radicals (such as the generalized species $A^\cdot$ and $B^\cdot$ in the reaction above) not only in their charge, but in their often reduced tendency to react together. The superoxide ion ($O_2^{\cdot-}$) is no exception, being much less reactive in the sense of bond making and breaking, than its conjugate acid $HO_2^\cdot$. Nevertheless, superoxide is sufficiently reactive and dangerous to biological systems that there is a natural enzyme, superoxide dismutase, specifically to keep the concentration as low as possible.

Superoxide ions are readily formed from oxygen by electron addition, but can also be generated by the $Ti(III)-H_2O_2$ system used by Ragai in the following reactions, where $Ti(III)$ acts as a powerful electron donor and H_2O_2 reacts by undergoing bond fission, the electron staying with one half to form the stable hydroxide ion, leaving the other as the highly reactive $\cdot OH$ radical. This is not detected as such by electron spin resonance, but readily extracts a hydrogen atom from H_2O_2 to give $HO_2^\cdot$. In neutral aqueous systems this largely ionizes to give $O_2^{\cdot-}$



Ragai's technique makes use of the fact that $Ti(IV)$ readily forms highly crosslinked gels comprising chains with



linkages as a backbone, and it seems that $O_2^{\cdot-}$ (but probably not $HO_2^\cdot$ or $\cdot OH$) can be trapped therein, in remarkably stable

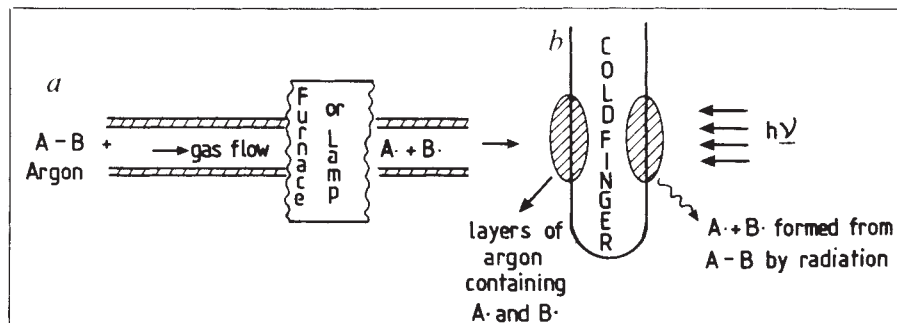


Fig. 1 Typical examples of isolating and thereby stabilizing reactive radicals by trapping in solids (matrix isolation). *a*, $A^\cdot$ and $B^\cdot$ radicals formed in flowing inert gas by thermolysis or photolysis followed by rapid solidification on a liquid helium cold finger. *b*, The same radicals generated *in situ* by photolysis or ionizing radiation (of energy $h\nu$).

sites. Thus they withstand heating to 110 °C and remain unchanged after 2 years at Egyptian temperatures.

Two aspects of these results indicate the nature of these sites. One is the electron spin resonance spectrum and, in particular, the low value of $g_{\max}$, g being a measure of any orbital magnetic contributions. To explain the significance of this, recall that 'free' O_2^- has its unpaired electron in an unusual orbital in that it rotates around the axis of the dioxygen unit, giving a maximal orbital contribution. This rotation makes it impossible to detect O_2^- by normal electron spin resonance. It is possible, however, to quench this orbital rotation by various interactions. A revealing experiment involves electron addition to normal oxygen dissolved in an alcohol which has been frozen to about 4 K in liquid helium. (The electrons were generated by ionizing radiation: Symons, M.C.R. & Stephenson, J.M. *Trans. Faraday Soc.* **1**, 1579–1583; 1981.) No spectrum for O_2^- ions could be detected at 4 K, but on warming to about 77 K a well-defined spectrum was obtained, characteristic of fully solvated

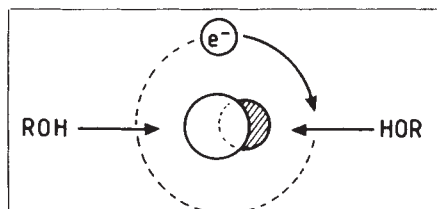
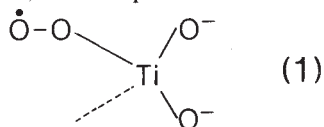


Fig. 2 Effect of hydrogen bonding from ROH molecules on the orbital motion of the unpaired electron in O_2^- .

O_2^- ions. The key to this result is selective hydrogen bonding or solvation by the alcohol which is confined to one plane, thereby preventing orbital motion (Fig. 2). Even so, the resulting value of $g_{\max}$ is about 2.08, not 2.00, the value expected if orbital motion were impossible. Now, O_2^- in the TiO_2 gel system has $g_{\max} = 2.02$, which is far closer to 2.00, and hence some sort of bonding which is stronger than normal solvation has to be invoked. This is almost certainly bonding to a $Ti(IV)$ centre, as, for example



(If this idea is correct, then an extra fingerprint should be detectable at high instrument gain. That is, electron–nuclear hyperfine coupling to the low-abundant isotopes of titanium having magnetic nuclei. This cannot be seen in the published spectrum, but might be detectable at high gain.)

Further evidence for a structure of this sort comes from Ragai's infrared results. Free O_2^- ions do not absorb infrared radiation because of their high symmetry, but the O–O bond in unit (1), being asym-

metrical, is expected to absorb, as was indeed the case. If this is correct, the species is perhaps better described as a peroxy-titanium radical rather than a superoxide ion.

It has only recently been realized that radical trapping has been occurring in nature for many millions of years. Most hard, non-metallic, geological materials, such as flint or calcite, form radical species which are permanently trapped within the solid on exposure to ionizing radiation. As there is always a low level of natural radiation, these trapped species are formed naturally and their concentrations grad-

ually increase. Electron spin resonance spectroscopy can often be used to detect, characterize and estimate the yields of these centres (see, for example, Robins, G.V., Seeley, N.J., Symons, M.C.R. & McNeil, D.A.C. *Nature* **276**, 103–106; 1978). Also, many flints have been shown to contain trapped methyl radicals ($CH_3^\bullet$) which are normally extremely reactive (Griffiths, D.R. *et al.* *Nature* **300**, 435–436; 1982). □

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T-cell receptors

Who needs more?

Ellis L. Reinherz

THYMUS-derived (T) lymphocytes comprise approximately 60–80 % of peripheral blood lymphoid cells and serve as both effectors and regulators of the vertebrate immune system. Unlike B lymphocytes, which produce immunoglobulin molecules acting to neutralize antigen in solution, T lymphocytes express membrane-bound receptors that detect perturbations in normal cell surfaces consequent upon invasion by viruses, certain bacteria and many intracellular parasites. Therefore, the elucidation of the structural basis for T-cell recognition is of basic theoretical and practical significance. On pages 683 and 689 of this issue^{1,2}, two groups report on the definitive identification of several additional types of T-cell receptors that differ in certain fundamental ways from the now classical T-cell receptor described three years ago^{3–6}.

Although this new set of receptors represents only a minor portion of peripheral T-lymphocyte receptors (less than 5 %), their early appearance in ontogeny and apparently broader specificity than conventional T-cell receptors, together with the absence of any obvious requirement for major histocompatibility complex (MHC)-restricted recognition (see below), thus defines a set of naturally occurring receptor variants that may provide insights into basic principles of T-cell receptor recognition in general. To put these findings in perspective, I shall first briefly review some of the salient features of T-cell recognition and its known structural basis.

In both human and murine systems, the subset of T cells that turns on the immune response, helper T cells, and the T cells responsible for specific destruction of target cells, cytotoxic T lymphocytes, express a T-cell receptor that is a molecular complex comprising at least five polypeptide chains^{7,8}. Two of these are the highly variable disulphide-linked $Ti\alpha$ and β

subunits, each encoded by a gene assembled by means of somatic rearrangement from pools of separate gene segments⁹, as with immunoglobulin genes (see Fig. 1 and legend); together these two chains mediate antigen recognition. They are non-covalently associated with the three monomorphic CD3 subunits that are believed to mediate signal transduction when the T cells are activated by receptor binding. All these chains have been cloned and sequenced (see ref. 10 for review).

A fundamental feature of most CD3– $Ti\alpha\beta$ -type T-cell receptors is that they recognize antigen only when it is associated on the surface of a cell with a molecule encoded by the MHC¹¹. Thus, recognition of either a specific MHC molecule or nominal antigen alone will not be sufficient to trigger activation of a given T lymphocyte.

Note on nomenclature

In the interests of consistency, *Nature* will in future use the CD nomenclature for all T-cell differentiation antigens between CD1 and CD8. Some of the alternative designations for man and mouse are given in the table.

Preferred name	Man	Mouse
CD1	T6/Leu 6	TL
CD2	T11/Leu 5	—
CD3	T3/Leu 4	—
CD4	T4/Leu 3	L3T4
CD5	T1/Leu 1	Ly-1 (Lyt-1)
CD8	T8/Leu 2	Ly-2,3 (Lyt-2,3)

The CD usage has thus been imposed on this News and Views article as well as the papers to which it refers. This convention will however not be extended to antigens whose alternative designations convey information about the function of the molecule: the interleukin-2 receptor, for example, should not be called CD25. □

phocyte. This so-called MHC restriction is 'learned' or selected for during T-lymphocyte ontogeny in the thymus^{12,13} and probably does not represent a structural limitation of the Ti $\alpha\beta$ heterodimer, as it is possible for some Ti $\alpha\beta$ heterodimers to recognize target structures without binding to MHC¹⁴. What then are the nature and function of the 'alternative' types of T-cell receptor?

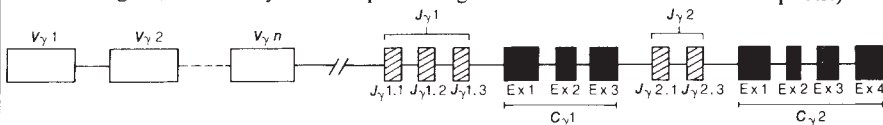
The new receptor complex

Several months ago, four groups independently reported on the identification of a CD3 complex that did not contain the Ti $\alpha\beta$ heterodimer. The complex was detected on cell lines established from immunodeficiency patients¹⁵; clones established from fetal blood¹⁶ and thymocytes¹⁷; and a leukaemia T-cell line¹⁸. All these cells express CD3 in association with a dimer containing a product derived from the γ gene, a gene essentially similar in structure to those encoding the antigen receptors of T and B cells, although with significantly more limited variability than α or β ¹⁹. This was the first direct evidence that the γ gene encoded a surface receptor. Unlike the genes encoding the α and β chains, whose products had been identified as variable cell-surface molecules responsible for specific target recognition by human T cells²⁰, the γ gene was isolated from murine DNA in the absence of any evidence on its product, and its identification as a receptor gene rested largely on its structure and the fact that it is rearranged during T-cell ontogeny (see Fig. 1 and legend).

One obvious question was therefore why, if the γ gene encodes a variable surface receptor for antigen, no γ chain had ever been identified by the techniques successfully used to isolate the α and β chains. A possible answer is provided by estimates of the proportion of cells in normal lymphoid tissue that express the alternative receptor, which seems to be present on only about 3 % of human peripheral T cells and as few as 0.2–0.9 % of neonatal human thymocytes²¹.

Moreover, almost all of these cells lack the CD4 and CD8 markers that characterize mature functional T cells (see Fig. 2 and legend; rare exceptions express CD8). This apparently very immature phenotype raises the question of whether these cells have a normal function in the mature immune system and has refuelled speculation, based on earlier observations in mice, that γ might instead have a special role early in ontogeny. Because γ gene transcription is particularly pronounced in immature T-lineage cells, such as fetal thymocytes, Tonegawa and colleagues proposed that the γ protein might associate with the β chain to give specificity for self-MHC, and thus allow for selection of self-MHC restricted cells during thymic ontogeny²². More recent

Fig. 1 Genomic structure and organization of the human Ti γ gene^{23,29,30}. The organization of the γ gene is like that of all the known genes encoding lymphocyte receptors for antigen, in that it comprises separate segments that are brought together in the course of lymphocyte ontogeny by DNA rearrangements. In all cases, there are relatively few invariant or constant segments (C) that anchor the receptor in the membrane, and sometimes mediate an effector function, and a linked pool of two or three additional segments that together encode the variable (V) region of the receptor that recognizes antigen. The largest of these segments is the V segment, of which there may be a very large and diverse pool; in the course of ontogeny one of these V segments becomes joined to one of a smaller number of J segments. (Some receptor chains also have a D region, encoded by a further pool of segments in between the V and the J pools.)



The human γ locus^{29,31} contains two constant regions, C₁ and C₂, each with several J_γ segments, as well as at least nine V segments that can recombine independently with either C_γ gene. Although the human C₁ segment, like both of the mouse C_γ segments, has three exons (Ex), in the human C₂ segment the second exon is duplicated and both copies have lost the codon for the cysteine residue thought to be involved in interchain disulphide bond formation. In PEER and IDP2 cells, which have the non-disulphide-linked receptor, complementary DNA analysis suggests that the C₂ segment is used (ref. 32 and Krangel and Brenner, personal communication). There seems to be some variation in the duplicated C₂ exons from different cell lines³³ but whether these differences are due to distinct C₂ alleles, alternative RNA splicing or utilization of other C_γ segments yet to be defined is not known. (In mouse, by contrast, both C_{γ1} and C_{γ2} encode cysteines.)

analyses suggest a rather different role for γ in T-cell ontogeny (see later); and although a role in thymic education cannot be excluded, the proposal originally formulated by Tonegawa and co-workers is almost certainly incorrect in the light of what is now known about the biochemistry and pattern of expression of the γ receptor complex.

The nature of the receptor

It is clear from the two reports in this issue^{1,2} that there are at least two forms of the 'new' receptor. Borst and colleagues¹ report a disulphide-linked dimer of relative molecular mass (M_r) 70,000–80,000 (70–80K) associated with CD3 and reacting with anti- γ antibodies on the surface of cloned human peripheral T cells. After separation of the chains in reducing conditions, the γ component resolves into two bands at 40 and 36K. These have the same isoelectric focusing point and reduce to a single major component after endo-F treatment, and so seem to represent differently glycosylated forms of the γ gene product¹. Borst *et al.* also detect a third component of M_r 43K that does not react with anti- γ antisera and that presumably forms a heterodimer either with the 40K or with the 36K γ chain.

This means that the receptor they now report is significantly different from the one originally described by Brenner and Krangel¹⁵. That receptor was a heterodimer from a cell line (IDP2) derived from an immunodeficiency patient and containing a 55–60K γ chain non-disulphide-bonded to a 40K non- γ product; they now find² a similar non-disulphide-linked form of the receptor on the leukaemic cell line PEER. This is clearly distinct from the receptor described by Borst *et al.*, both in the absence of the disulphide bonding and in the molecular mass of the non- γ component;

however, Brenner and Krangel also report, on a human T-cell clone, a disulphide-linked version containing 36K and 40K γ components as described by Borst *et al.* For reasons that are not clear but are probably trivial, they do not detect the 43K component on their gels; but they postulate a second, non- γ component because of the very different migration of the CD3-associated bands in isoelectric focusing gels under reducing and non-reducing conditions. Brenner and Krangel have termed the non- γ component in these receptors δ ; but because its relationship to non- γ components described by Borst *et al.* and other workers is unknown, I shall here refer to all non- γ components as X (see Fig. 2).

Whatever the nature of X, it is clear on grounds of disulphide linkage and of the molecular mass of the γ component that there are two distinct forms of the receptor. Moreover, the discrepancy in molecular mass of γ products in the non-disulphide-linked and disulphide-linked forms cannot be explained away by differences in glycosylation: endoglycosidase digestion reveals a substantial core protein size difference¹. In fact, there is now strong evidence that both the size difference and the absence of disulphide bonding can be explained by differential usage of the two human C_γ gene segments (see Fig. 1): C₂ usage correlates with the expression of the non-disulphide-linked CD3-associated form, whereas C₁ usage results in formation of the disulphide-linked Ti γ X heterodimer. Both forms appear in peripheral blood lymphocytes from normal humans. At this stage it is impossible to guess at the functional significance of the distinct forms — especially as mice do not seem to produce the non-disulphide-linked form, having cysteines in both C_{γ1} and C_{γ2}.

What is the alternative receptor for?

Although the definitive word is not yet in, there is evidence that both forms of the CD3-Ti γ X receptor mediate non-MHC-restricted killing of target cells. First, Borst *et al.* report¹ that cells bearing the disulphide-linked receptor display cytotoxic activity against a wide array of target cells. Second, Krangel and Brenner have data of a similar kind for clones bearing either the disulphide-bonded or the non-disulphide-bonded receptor and find that cytotoxicity is not blocked by antibodies against MHC gene products. This sets them apart from the classical cytotoxic T lymphocytes which must recognize MHC products in order to kill, and places them in a rather broadly defined category of lymphocytes with generalized cytotoxic activity that does not (again unlike that of classical cytotoxic cells) require prior sensitization and that is directed against a broad range of targets. Such killing may be targeted by antibodies bound to the surface of a cell (antibody-dependent cell-mediated cytotoxicity, or ADCC); or not, in which case it is called natural killing. Most 'natural killer' cells (possibly all) are also capable of ADCC: all the clones tested by Borst *et al.* show both activities.

Because it is possible to elicit a more-or-less indiscriminate wide range of cytotoxic responses from such T-lineage cells in the absence of identifiable receptor binding, it is a little difficult to know what to make of the broad-spectrum cytotoxicity of the γ X-bearing cells. There is, however,

some direct evidence, from studies by Moingeon *et al.* reported in this issue²³, that the CD3-Ti γ X complex is a receptor for non-MHC-restricted recognition. These authors have produced specific antibodies against a T-cell clone (F6C7) which expresses a disulphide-linked receptor containing γ bonded either to another γ or to some other chain; and they are able to show that such antibodies can induce proliferation and the production of interleukin-2 by these cells, or block killing, depending on the conditions. That γ -bearing cells can kill in the absence of MHC recognition is not however a powerful argument against a more specific MHC-restricted cytotoxic function for at least some of them. Recall that CD3-Ti $\alpha\beta$ receptors can recognize antigen in the absence of MHC in the case of certain natural killer clones¹⁴. The prominent non-MHC restricted cytotoxicity documented in recent reports may be more a consequence of the ease with which immunologists can detect non-MHC-restricted killing than any limitation of the recognition repertoire of the CD3-Ti γ X receptor.

Although functional and biochemical analyses have proceeded more rapidly in human systems, studies on the ontogeny of γ expression have made more progress in mice. The character of the receptor and of the cells that bear it are essentially the same in mouse as in man. A 35K γ chain, disulphide-linked to a 45K partner associated with CD3, is found on cells not expressing either of the murine equivalents of CD8 (L3T4) and CD4 (Ly 2), and the

cells can be stimulated to proliferation and cytotoxicity by interleukin-2 and antibodies against CD3 (refs 24-27). However, it has been possible to show clearly in mice that the Ti- γ X complex is predominantly a fetal receptor.

Coligan, Pardoll, Bluestone and associates²⁷⁻²⁹ have demonstrated that at day 15 of gestation, only the γ X proteins are associated with CD3 on the surface of thymocytes. By day 17.5, there are about equivalent amounts of γ X and $\alpha\beta$ associated with thymic CD3; and by birth on day 20, no γ X is detectable in unfractionated thymocytes.

On these data alone, the idea that the γ product is expressed early in ontogeny in precursors to the $\alpha\beta$ -bearing cells is still tenable. But for several reasons it now seems much more likely that the γ -bearing cells comprise a separate T-cell lineage whose intrathymic development precedes that of cells expressing CD3-Ti $\alpha\beta$.

First, the γ protein on fetal and adult thymocytes bears N-linked carbohydrates and is therefore presumably derived from the $C_{\gamma}1$ gene segment which, unlike $C_{\gamma}2$, has sites for these glycans. This is significant because most mature $\alpha\beta$ -bearing T cells contain only defective (out of frame) transcripts from the $C_{\gamma}2$ segment. Second, it has been possible to identify γ -bearing cells in the chicken, where the stages of fetal development are exceptionally well defined, and show that the precursor cells that migrate to the thymus seem to acquire Ti γ X early in ontogeny but Ti $\alpha\beta$ later. Cooper and Chen (personal communication) have produced monoclonal antibodies against the chicken CD3, CD4 and CD8 structures as well as one against a receptor determinant (TCR1) that identifies a putative Ti γ epitope expressed on CD3⁺CD4⁺CD8⁻ T-lineage cells in the chick and precipitates a disulphide-linked CD3-associated heterodimer with subunits of M_r 50K and 38K.

During fetal development precursor cells move into the thymic epithelium in discrete waves. The first wave, which is initially CD3⁻, rapidly expresses CD3 and TCR1 but not CD4 or CD8. The CD3⁺TCR1⁺ cells account for 30 % of thymocytes at embryonic day 15, but drop to less than 5 % by the day of hatching (day 21), while the frequency of CD3⁺CD4⁺CD8⁺TCR1⁻ cells increases. Subsequent waves of thymic progenitors contain much smaller number of cells expressing TCR1, although they acquire CD3 and thus probably also Ti $\alpha\beta$ (as CD3 and Ti are believed to be obligatorily co-expressed). In the adult chicken, 10 % of thymocytes and 25 % of peripheral T cells react with TCR1. The data thus suggest that the CD3⁺Ti γ cells are a separate sub-lineage rather than a precursor of the CD3-Ti $\alpha\beta$ -receptor expressing cells.

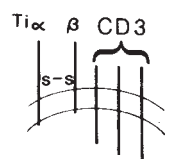
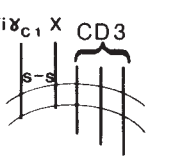
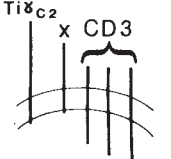
Receptor subunits			
Distribution	≥95% T cells	≤5% T cells	≤5% T cells
Phenotype	CD3 ⁺ CD4 ⁺ CD8 ⁻ CD3 ⁺ CD4 ⁻ CD8 ⁺	CD3 ⁺ CD4 ⁻ CD8 ⁻	CD3 ⁺ CD4 ⁻ CD8 ⁻
Ontogeny	later	earlier	earlier
Function	T _H , T _C , T _{NK} , ?T _S	NK-like, ?other	NK-like, ?other

Fig. 2 Phenotype and pattern of expression of T-cell receptors for antigen. The receptor dimers (Ti) are shown in association with the three CD3 subunits, CD3 γ , CD3 δ and CD3 ϵ , referred to collectively as CD3. The Ti γ partner, which has been termed δ by some, is denoted here as X to acknowledge the possibility that it represents an unknown C_{γ} product and to avoid confusion with the CD3 δ subunit (note that there is already scope for confusion between Ti γ and CD3 γ). Possible additional subunits are not included³⁴. Also note that the less commonly identified CD3⁺CD4⁺CD8⁺Ti γ X phenotype is not shown. Other cell-surface molecules defining the phenotype are designated CD4 and CD8. Their function is not known for sure but they are strongly implicated in enhancing the binding of the T-cell receptor to MHC molecules. As a rule, CD4 performs this function on T helper cells (generally MHC class II-restricted) and CD8 on cytotoxic cells (generally MHC class I-restricted). Neither of these molecules is present on the surface of T-lineage cells early in thymic development, but it is believed that the precursors to mature functional T cells expressing the Ti $\alpha\beta$ receptor express either or both.

It is far from clear what regulates differentiation along CD3-Ti γ X as against CD3-Ti $\alpha\beta$ pathways. However, CD3-Ti γ X clones generally have one functional γ gene rearrangement and usually no β gene rearrangements whereas, in contrast, CD3-Ti $\alpha\beta$ clones have several non-functional γ gene rearrangements and functional β gene rearrangements, so that a stochastic model may be applicable. Thus, if γ gene rearrangement is productive, productive β and α gene rearrangement fails to occur. Alternatively, if γ gene rearrangement is unsuccessful in both alleles, rearrangement attempts continue. Nevertheless, such a model does not readily explain why CD3-Ti γ X as opposed to CD3-Ti $\alpha\beta$ expression is mostly limited to thymocytes of the CD4⁺CD8⁻ phenotype.

Why have several forms of T-cell receptor? Certainly, CD3-Ti γ X receptors lack the extensive germline diversity of their CD3-Ti $\alpha\beta$ counterparts, implying that the specificities they encode are more limited. Could this receptor complex have been the phylogenetic precursor of the $\alpha\beta$ heterodimer? If so, it is far from vestigial, given the precise maintenance of recombination-recognition signals in the various Ti γ gene segments and the nature of the γ gene protein.

There seem to be two other possibilities, not mutually exclusive. The CD3-Ti γ X receptor that appears early in ontogeny may provide protective immunity to the fetus against common viral or other pathogens before the development of MHC-restricted CD3-Ti $\alpha\beta$ receptor structures. In the mature peripheral compartment, the CD3-Ti γ X receptor may encode specificities not provided by the CD3-Ti $\alpha\beta$ repertoire. This would make it perhaps analogous to the minor light chains of immunoglobulins, which although less diverse than the major class nonetheless make a significant contribution to total antibody diversity. It is worth noting that the δ subunit of the CD3 complex associated with γ X has a different electrophoretic mobility from its counterpart in the CD3-Ti $\alpha\beta$ receptor^{1,2}. It remains to be determined whether this difference is due to glycan or peptide. But either could signify differences in the physiology of T-cell activation by the different receptors. Differences in refractoriness or sensitivity to triggering events may be important in functionally diverse and perhaps ontogenetically distinct populations, such as suppressor T lymphocytes.

Finally, there is the obvious outstanding question of the origin of the Ti γ -associated X chain. Is this also a structure with a variable domain encoded by a rearranging set of genes? Variability in peptides should be detectable by comparison of X chains from different clones, as it was in the earlier investigations on the Ti α and β subunits. Identification of X will

elucidate whether Ti γ associates only with X or whether there are still other chains yielding still more types of receptor. It is possible that the recently identified murine C₄ locus²⁸, which is only weakly homologous to the other C_γ loci and which contains N-linked glycosylation sites, may encode a Ti γ partner. □

1. Borst, J. *et al. Nature* **325**, 683 (1987).
2. Brenner, M.B. *et al. Nature* **325**, 689 (1987).
3. Allison, J.P., McIntyre, B.W. & Bloch, D. *J. Immun.* **129**, 2293 (1982).
4. Meuer, S.C. *et al. J. exp. Med.* **157**, 705 (1983).
5. Acuto, O. *et al. Cell* **34**, 717 (1983).
6. Haskins, K. *et al. J. exp. Med.* **157**, 1149 (1983).
7. Meuer, S.C. *et al. Science* **222**, 1239 (1983).
8. Borst, J., Prendiville, M.A. & Terhorst, C. *J. Immun.* **128**, 1560 (1982).
9. Hedrick, S.M. *et al. Nature* **308**, 153 (1984).
10. Robertson, M. *Nature* **317**, 768 (1984).
11. Benacerraf, B. & McDevitt, H.O. *Science* **175**, 273 (1972).
12. Zinkernagel, R.M. & Doherty, P.C. *J. exp. Med.* **141**, 1427 (1975).
13. Hunig, T. & Bevan, M.J. *Nature* **294**, 460 (1981).
14. Ritz, J. *et al. Science* **228**, 1540 (1980).
15. Brenner, M.B. *et al. Nature* **322**, 145 (1986).

16. Moingeon, P. *et al. Nature* **323**, 638 (1986).
17. Bank, I. *et al. Nature* **322**, 179 (1986).
18. Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998 (1986).
19. Saito, H. *et al. Nature* **309**, 757 (1984).
20. Marrack, P. *et al. J. exp. Med.* **158**, 1635 (1983).
21. Lanier, L.L. & Weiss, A. *Nature* **324**, 268 (1986).
22. Raulat, D. *et al. Nature* **314**, 103 (1985).
23. Moingeon, P. *et al. Nature* **325**, 723 (1987).
24. Lew, A.M. *et al. Science* **234**, 1401 (1986).
25. Bluestone, J.A., Pardoll, D., Sharrow, S.O. & Fowlkes, B.J. *Nature* (in the press).
26. Pardoll, D.M. *et al. Nature* (in the press).
27. Nakanishi, N., Maeda, K., Ito, K., Heller, M. & Tonegawa, S. *Nature* **325**, 720 (1987).
28. Iwamoto, A. *et al. J. exp. Med.* **163**, 1203 (1986).
29. LeFranc, M.P., Forster, A., Baer, R., Stinson, M.A. & Rabbitts, T.H. *Cell* **45**, 237-246 (1986).
30. Quertermous, T., Strauss, W.M., van Dongen, J.J.M. & Seidman, J.G. *J. Immun.* (in the press).
31. LeFranc, M.P., Forster, A. & Rabbitts, T.H. *Proc. natn. Acad. Sci. U.S.A.* **83**, 9596 (1986).
32. Littman, D. *et al. Nature* (in the press).
33. Dialynas, D.P. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 2619 (1986).
34. Samelson, L., Harper, J.B. & Klausner, R.D. *Cell* **43**, 223 (1985).

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Photosynthesis

Structure of key enzyme refined

Jim Barber

DETERMINATION of the structure of the leaf protein ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) is likely to be an important step for improving crop productivity by increasing the efficiency of photosynthesis. On page 730 of this issue¹ Holzenburg *et al.* identify the conformational changes that occur when substrate binds to the higher-plant form of this enzyme, an important result for future attempts to 'engineer' crop efficiency.

RuBPCase, the most abundant naturally occurring enzyme², initiates the reduction of atmospheric carbon dioxide to organic molecules during photosynthesis. Every molecule of fixed carbon in the biosphere originates from its catalytic activity, and it catalyses the fixation of about 10¹¹ tons of CO₂ per year. Despite the key role of this enzyme, it does not work at maximum efficiency. One reason for this is that it catalyses both the carboxylation of ribulose-1,5-bisphosphate and its oxygenation³. The oxygenase activity results in photorespiration, which substantially reduces the overall rate of photosynthesis and consequently plant productivity.

It has frequently been argued, therefore, that it will be necessary to reduce or eliminate the oxygenase activity of RuBPCase to improve the efficiency of photosynthesis. Some plants, called C₄ plants, overcome the carboxylase/oxygenase problem by increasing the relative level of CO₂ at the reactive site of the enzyme⁴. In general these plants are tropical or subtropical species, such as sugar cane. Most plants growing in temperate climates, however, are C₃ type which use only the

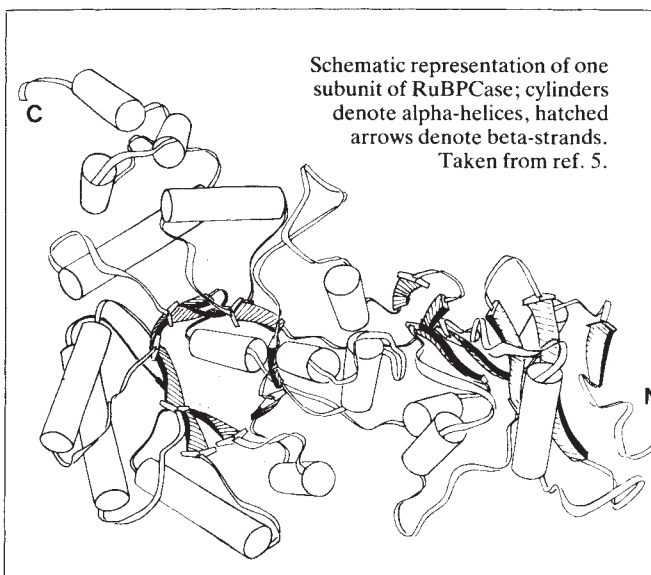
reductive pentose phosphate cycle to fix carbon and therefore support significant levels of photorespiration. At present, the only method for increasing their productivity is to grow these plants in an atmosphere enriched with CO₂, a valuable procedure in the horticulture industry, where greenhouses are used for cultivating commercially important crops. But for crops grown in the field, and therefore subjected to atmospheric concentrations of CO₂, the hope for increasing photosynthetic efficiency is to produce new varieties of plants containing RuBPCase with reduced amounts of oxygenase activity. Attempts to isolate mutants with this preferred characteristic have so far been unsuccessful and the only solution seems to be the application of *in vitro* genetic engineering using recombinant DNA techniques. To achieve this, however, will require a complete understanding of the catalytic mechanism of the enzyme in terms of its molecular structure.

RuBPCase in higher plants and in a range of other organisms, including algae and photosynthetic bacteria, consists of eight small and eight large subunits (L₈S₈). In higher plants and algae, the S subunits are encoded by the nucleus whereas the L subunits are encoded by the chloroplast genome. It is known that the catalytic site is located on the L subunits and that a specific lysine residue becomes carbamylated by an activator CO₂ molecule and stabilized by a magnesium ion. This activation process is necessary for both carboxylation and oxygenation reactions. The activated ternary complex can react with the substrate ribulose-1,5-

bisphosphate, which is subsequently attacked either by CO_2 or by O_2 . All the biochemical evidence indicates that both carboxylation and oxygenation reactions occur at the same site in the protein.

Significant recent advances have been made in the attempt to link the enzymology of RuBPCase with its structure. Schneider *et al.*⁵ have successfully obtained the three-dimensional structure to a resolution of 2.9 Å of a simpler form of the enzyme, consisting of only two large subunits (L_2) and isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (see figure). These authors showed that the active site is located in a carboxy-terminal domain which has an eight-stranded parallel alpha/beta-barrel structure. The three lysine residues in the site are in loops at the carboxy ends of the beta-strands, regions which have very similar amino-acid sequences to the enzyme in higher plants. Although the L_8S_8 form of the enzyme in higher plants was crystallized several years ago⁶, its large relative molecular mass (550,000) and its complex structure have hampered analyses of data obtained by X-ray diffraction.

Various techniques indicate that the L_8S_8 complex has a diameter of 124 Å and a height of 100 Å, and Holzenburg and colleagues in this issue¹ now present the structure of the L_8S_8 form in more detail. They isolated the enzyme from the hydrogen-oxidizing bacterium *Alcaligenes eutrophus* and crystallized it in its activated state with and without the intermediate substrate 2-carboxyarabinitol-1,5-bisphosphate (CABP). The authors used X-ray crystallography and electron microscopy to obtain structural information to a resolution of 5 and 17 Å, respectively. Both methods give comparable results and show that binding of the substrate results in a new structural state of the enzyme. The L_8S_8 complex consists of two L_4S_4 halves, each with 4-fold symmetry. After substrate binding, the 4-fold axes of the L_4S_4 halves no longer coincide, being shifted by 36 Å and related by a 2-fold axis



tially important amino acids within the protein it is necessary to know the organization of the L_8S_8 complex at a resolution of 3 Å or less. As mentioned above, the structure of the L_2 enzyme of *R. rubrum* has just been solved at this level of resolution⁸, and indeed its active site has already been modified by site-directed mutagenesis⁹⁻¹¹. It seems most likely, therefore, that for the time being work on the simpler bacterial L_2 form will lay the foundations for establishing whether it is possible to increase the carboxylation reaction of the enzyme while reducing its oxygenic activity.

The work of Holzenburg *et al.* does, however, have important implications regarding the diurnal variation of RuBPCase activity, which seems to be under the control of an inhibitor molecule very similar to the CABP substrate used in their structural studies. The identity of this natural reversible inhibitor was recently reported in *Nature*¹² by Gutteridge and colleagues, who showed it to be 2-carboxyarabinitol-1-phosphate. □

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perpendicular to, and between, the local 4-fold axes. The detection of this conformational change after substrate binding supports earlier X-ray work⁶ with the tobacco enzyme and biochemical studies⁷ with the spinach enzyme.

Unfortunately, the spatial resolution of the structure described by Holzenburg *et al.* is not sufficient to begin engineering the enzymic properties of RuBPCase. To pinpoint the exact positioning of poten-

Solid-state physics

Superconductivity at high temperatures in doped oxides

M. Strongin, D.O. Welch and J.W. Davenport *£55*

HIGH-temperature superconductivity has always been something of an oxymoron. Although there have never been any theoretical prohibitions against superconductivity at, for example, room temperature, it has always been a distinctly low-temperature affair: until very recently, superconductivity has been confined to temperatures less than 23 K, notwithstanding considerable theoretical effort exploring various exotic mechanisms and several experimental false alarms. But high-temperature superconductivity is a phenomenon of enormous importance and potential as it holds the possibility of resistanceless transmission of electrical power and the possibility of powerful electromagnets and generators that would operate without electrical resistance. Nonetheless, progress in raising the temperature T_c of the transitions to the superconducting state has, until now, been frustratingly slow, and in recent years the search for new high- T_c materials has lost impetus. The recent discovery of oxide superconductors with T_c values greater than 30 K has brought new enthusiasm to

superconductivity research.

The apparent barrier to T_c above 23 K has been lifted by the discovery of the $\text{La}_{2-x}\text{X}_x\text{CuO}_4$ superconductors (where X is, for example, Ba, Sr or Ca), and it seems reasonable that superconductivity in other similar compounds will be discovered; furthermore the real possibility exists for superconductivity at liquid-nitrogen temperatures and higher. The recent meeting on 20 January at the Argonne National Laboratory was held to exchange information and to coordinate the already active programmes in Department of Energy laboratories in the United States, and some of the experiments we discuss here were reported at the meeting. The technological ramifications are obvious for power transmission, magnets, superconducting electronics and other electrical devices, and there are already speculations about new magnets for planned accelerators. This is probably somewhat premature because of the length of time usually necessary to go from samples in the laboratory to the long lengths of wire necessary to wind magnets. But it is clear that this is

1. Holzenburg, A. *et al.* *Nature* **325**, 730-732 (1987).
2. Ellis, R.J. *Trends biochem. Sci.* **4**, 241-244 (1979).
3. Mizioro, H.M. & Lorimer, G.A. *Rev. Biochem.* **52**, 507-535 (1983).
4. Edwards, G.E., Ku, M.S.B. & Monson, R.K. in *Photosynthetic Mechanisms and the Environment* Vol. 6 (ed. Barber, J.) 287-327 (Elsevier, Amsterdam, 1985).
5. Schneider, G., Lindqvist, Y., Branden, C.-I. & Lorimer, G. *EMBO J.* **5**, 3409-3415 (1986).
6. Baker, T.S., Eiserling, F.A. & Weissmann, L.J. *J. molec. Biol.* **91**, 391-399 (1975).
7. Johal, S., Partridge, B.E. & Cholet, R. *J. biol. Chem.* **260**, 9894-9904 (1985).
8. Gutteridge, S. *et al.* *EMBO J.* **3**, 2737-2742 (1984).
9. Estelle, M., Hanks, J., McIntosh, L. & Somerville, C. *J. biol. Chem.* **260**, 9523-9526 (1985).
10. Niyogi, S.K. *et al.* *J. biol. Chem.* **261**, 10087-10092 (1986).
11. Terzaghi, B.E., Laing, W.A., Christeller, J.T., Petersen, G.B. & Hill, D.F. *Biochem. J.* **235**, 839-846 (1986).
12. Gutteridge, S. *et al.* *Nature* **324**, 274-276 (1986).

only a matter of time, and if the fuzzy reports of onsets near 70 K described below turn out to be true superconductivity, then electrical technology may truly be revolutionized in a few years.

The phenomenon of superconductivity was discovered in 1911 by Kammerlingh Onnes, who found that mercury became superconducting at about 4.2 K. In 1954, superconductivity in the A-15 compounds was discovered, with a T_c of 17.1 K in V_3Si , quickly followed by the discovery of Nb_3Sn with a T_c of 18.6 K. It then took 20 years until Nb_3Ge was discovered, and this compound set the previous limit for T_c of about 23 K. Until a few months ago there had been no substantial progress in raising T_c , notwithstanding a few false alarms, most notably the case of $CuCl$. Most of the researchers in this area had begun to have a mystical or cynical view that superconductivity above about 23 K was impossible, and there were various speculations about the instabilities limiting the highest possible T_c .

Given this history, it is probably not surprising that when the great event happened it was accepted rather gradually. The predecessor of the exciting new results was the discovery¹ of superconductivity in the mixed-valent perovskite-structure oxide $BaPb_{1-x}Bi_xO_3$ with a T_c of 13 K. Then Bednorz and Müller of IBM Zurich Research Laboratory found evidence² of a much higher T_c in a multiphased sample in the $Ba-La-Cu-O$ system. Even with this evidence of superconductivity at 'high' temperatures, most researchers did not get very excited, possibly because Bednorz and Müller did not strongly associate their resistive anomalies with true bulk superconductivity, and suggested that the high onset temperature was caused by two-dimensional fluctuations. Probably because of false alarms and unconfirmed reports of high-temperature superconductivity in other exotic systems, most workers in the field appeared wary. It seems that the work of Uchida *et al.*³ at the University of Tokyo, which both confirms and extends the work of Bednorz and Müller, and shows higher-quality samples with real transitions, finally found general acceptance when it was presented at the Materials Research Society meeting in Boston, 1–6 December.

Uchida *et al.* made the important step of showing that the high- T_c phase in the multiphased material of Bednorz and Müller was $La_{1-x}Ba_xCuO_{4-y}$ with $x \sim 0.1$, which has the layered perovskite K_2NiF_4 structure. This crystal structure can be viewed as a stacking of $LaO-CuO_2-LaO$ sandwiches. It has been suggested that the states responsible for the superconductivity probably lie in the central CuO_2 plane. These states should be essentially two dimensional because the distance between CuO_2 planes is quite large (more than 6 Å). Whether this two dimensionality is

crucial to superconductivity is not known, but in recent years there has been some interest in low-dimensional systems such as the organic superconductors, which in some ways are one dimensional.

Since the beginning of December 1986 there have been rapid developments, which have been announced in several newspaper accounts and press conferences. C.W. Chu and co-workers at the University of Houston have reported onset of superconduction near 70 K and sharp regions in the transition as high as 60 K, and they show⁴ that the transition in $La-Ba-Cu$ -oxide can be enhanced by pressure. R.S. Cava *et al.* at AT&T Bell Laboratories, on the other hand, have substituted Sr for Ba and obtain⁵ sharp transitions as high as 38 K. In the meantime, workers in China (Z. Zhao *et al.*, Chinese Academy of Sciences preprint) have reported onsets near 50 K and full transitions above 40 K in both Sr- and Ba-doped $LaCuO_3$ with some evidence of onsets near 70 K. Similar results have been obtained in several other laboratories (for example, ref. 6).

One exciting prospect of such a large increase in the record value of T_c is that it could be the consequence of an exotic new mechanism, such as excitonic or bipolaronic mechanisms, for the interactions between electrons which leads to the formation of Cooper pairs, and hence superconductivity. But, the values of various characteristic superconducting parameters obtained so far for the new material indicate that although the parameters might be somewhat unusual, the superconductivity is caused by the usual electron-phonon interaction mechanism. Three of the parameters are the density of electronic states at the Fermi level, the electron-phonon coupling strength and the frequency of the interacting phonon modes. The density of states appears to be lower than in previous high-temperature superconductors, such as Nb_3Sn , but apparently the low density of states is more than compensated for by the large electron-phonon coupling.

As well as the vast array of conventional measurements in progress on the magnetic and electrical properties of these superconductors, microscopic probes are being used to elucidate geometrical and electronic structure of the superconducting phase. The aim of this work is to try to understand the fundamental mechanism for the superconductivity and to test the theoretical ideas that are already appearing, for example, the possible quasi-two-dimensional nature of the relevant electron bands and the role of a concomitant Peierls transition. Inelastic neutron-scattering experiments are already planned that will address the question of whether 'soft-phonon' modes dominate the electron-phonon coupling, and this work should be well under way by the time

this article is published. X-ray scattering experiments at the new generation of synchrotrons are also active, and should reveal structural instabilities in these complex crystal structures. The instabilities should help to explain how the superconductivity is related to the structure. Further X-ray absorption experiments are probing the CuK edge to examine the valence state of the copper in the oxide. Other experiments are even using muons to probe the magnetic-field distribution, and have already shown how the magnetic field falls off into these materials.

The sceptics may question whether these compounds truly exhibit superconductivity, or just show a transition from one metallic state to a metallic state that is three or four orders of magnitude more conducting. In other words, is this truly a superconductor with zero electrical resistance? Although the initially reported work has not yet addressed this point, the Bell group has measured⁷ the Meissner effect in these samples, and so have Bednorz and Müller⁸. To the aficionados, this is a true indication of superconductivity, as it implies that the sample expels magnetic flux when cooled in a magnetic field. A highly conducting metal will not do this, only a true superconductor. Although persistent currents and zero resistance have not yet been demonstrated, it is almost certain that this is true superconductivity and that persistent currents exist.

The future for the new high- T_c oxide superconductors appears bright, but an assessment of their potential usefulness requires the accurate determination of some crucial parameters. For example, to be useful in high-field magnets, the material must remain superconducting in high magnetic fields while carrying large amounts of electric current. Although values of the critical currents (the current above which the sample returns to the normal state) still appear small, it is almost certain that a large part of this results from the sample condition and imperfections. Even if the critical current turns out to be intrinsically small, this does not preclude a very useful role in superconducting electronics. Another important parameter is the critical magnetic field (the magnetic field above which the sample returns to the normal state), and although there is huge variation in the initial estimates, it seems that the critical field at 0 K can be greater than 50 tesla (compared with ~ 30 tesla for Nb_3Sn). □

1. Sleight, A.W., Gillson, J.L. & Bierstedt, F.E. *Solid State Commun.* **17**, 27 (1975).
2. Bednorz, J.G. & Müller, K.A. *Z. Phys.* **B64**, 189 (1986).
3. Uchida, S., Takagi, H., Kitazawa, K. & Tanaka, S. *Jap. J. appl. Phys. Lett.* (in the press).
4. Chu, C.W. *et al.* *Phys. Rev. Lett.* **58**, 405 (1987).
5. Cava, R.J., van Dover, R.B., Batlogg, B. & Rietman, E.A. *Phys. Rev. Lett.* **58**, 409 (1987).
6. Bednorz, J.G. & Müller, K.A. *Europhys. Lett.* **3**, 379 (1987).

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X-ray astronomy

Observations of active galaxies

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WITH the launch of the EXOSAT satellite in 1983 it became possible to look at the X-ray variability of active galactic nuclei in detail¹. The sensitivity and unprecedented capability of EXOSAT to perform continuous observations of up to 80 hours made it possible for the first time to characterize carefully the nature of the variability in a small but significant sample of 'normal' active galactic nuclei. Extremely high-quality observations are available for timescales from minutes to a day for more than ten active galactic nuclei and for timescales of up to 6 hours for many more; of the ten, at least six seem to show interesting variability. The first detailed analysis of these long looks is described in two papers on pages 694 and 696 of this issue².

One of the goals of the study of active galactic nuclei is the determination of fundamental properties such as mass, accretion rate and physical processes responsible for the emission. Simple theoretic

cal arguments suggest⁴ that these qualities can be derived from study of the X-ray variability. The fastest repeatable timescales should be related to the radius at which most of the energy of liberated. If the active galactic nuclei were accreting massive black holes, this would be of the order of 5 Schwarzschild radii⁵. The corresponding timescale is $\sim 50 M_6$ seconds, where M_6 is the mass of the central black hole in units of 10^6 solar masses. Longer characteristic times could be caused by heating/cooling of the radiating plasma or acceleration timescales for the particles.

A possible analogy between active galactic nuclei and the best-studied galactic black-hole candidate Cyg X-1 is intriguing. The X-ray light curve of Cyg X-1 is chaotic and often shows large amplitude variability. It appears to have two characteristic timescales, one of the order of milliseconds⁶, which is of the right order of magnitude for the 'observed' 10-solar mass black hole; and the other of the considerably longer timescale⁷ of about 1 second, which has never been convincingly explained.

Before the launch of EXOSAT, observations of X-ray time variability of active galactic nuclei could be understood as distinct flares superimposed on more-or-less consistent sources. These flares seemed to have well-determined doubling times; the best case was established for the very bright Seyfert 1 galaxy NGC4151 (refs 8,9). On shorter timescales most objects appeared not to show frequent large-amplitude variability¹⁰. The absence of data gaps and the quality of the data provided by EXOSAT allows detailed investigation of the form of the temporal variation of active galactic nuclei in a manner similar to studies of the galactic black-hole candidates Cyg X-1, GX339-4 and others^{2,3,11}.

The new EXOSAT results^{2,3} add considerable complexity and differ in two significant ways. First, many more objects are seen to vary on short timescales¹; and second, in some cases the character of the time variability is quantitatively and qualitatively different than expected from the flare 'model'. This is best seen in the power-density spectra of the variability in the two active galaxies NGC5506 and NGC4051 reported in this issue^{2,3}. These spectra are relatively featureless power laws of more than one decade in frequency f with a $\sim f^{-1}$ power spectrum. Such a spectrum is similar to that of turbulence and arises in many physical circumstances, but is poorly understood. Because the $\sim f^{-1}$ character of the power-density spectra

persists over the frequency range sampled, the quantity $F/(dF/dt)$, where F is source flux, is not uniquely defined, and "there are no preferred timescales"^{2,3}. In the case of a power-law power-density spectrum, the 'variability timescale' is model dependent and is a function of the signal-to-noise ratio and of observation length³. Thus, these results cast doubts on previous interpretations of the observed time variability in active galaxies¹², which indicated that there is a fundamental and simple relation between source luminosity and variability time. To confuse the situation further, another active galaxy, NGC4593, may not have a pure power-law spectrum¹ and autocorrelation analysis of this object gives sensible timescales which vary as the luminosity of the object, agreeing with the previously published correlation¹².

Previous reports¹³ on the time variability of NGC4051 used the autocorrelation function to demonstrate that this object had a characteristic variability timescale. Lawrence *et al.*² argue that as the power-density spectrum is the Fourier transform of the autocorrelation function, it must carry the same information and that there are problems in the interpretation of the function in the presence of low-frequency divergent noise. Of course, the spectrum cannot diverge to arbitrarily low frequencies because active galaxies do not in general show high-amplitude flux variability on long timescales^{14,15}. In particular the three objects I discuss here do not. The present two-day observations of NGC4051 and NGC5506 sample the entire known range in variability amplitude. This suggests, at least for these galaxies, that the characteristic variability timescale, the 'knee' in the power-density spectrum, is constrained to be less than a few days and no longer than for a few hours. Scaling of the observed knee in Cyg X-1 by the ratio of the luminosities would give a 'predicted' turnover in the power-density spectrum for NGC4051 of 5×10^{-6} Hz, which is just below the observational range from EXOSAT³. It is thus conceivable that the sought-for variability timescale is just longer than the low-frequency observational limit of EXOSAT.

The faintness of the sources and the relatively small size of the EXOSAT detectors do not yield sufficiently sensitive data to study the high-frequency ($> 10^{-3}$ Hz) regime. Models of Eddington-limited luminous active galaxies predict a frequency that is an order of magnitude higher than for the characteristic time of material near a few Schwarzschild radii. The Japanese X-ray astronomy satellite ASTRO-C, which will be launched this month, may have the sensitivity necessary to sample this high-frequency domain. The most interesting observations will be those that determine the frequency at which the sources cease to vary.

100 years ago



This is one of the most interesting books of travel that it has been our good fortune to meet with for several years ["The Cruise of the Marchesa to Kamschatka and New Guinea with notices of Formosa, Liu-Kiu and various islands of the Malay Archipelago" by F.H.H. Guillemard]. "Nearest us was Tolbatchinska; and beyond rose the twin peaks of Kojerevska and Kluchefskaya, pyramids of the purest snow, before which one felt how poor was all language to express the sense of their perfect beauty.

From *Nature* 35, 369; 17 February 1887.

This should be characteristic of the shortest length scales or the fastest physical processes.

To determine if the 'true characteristic' timescales are as long as suggested by the scaling of the 1-second time variability from Cyg X-1, long observations, of roughly 10 days' duration, will be required. As for Cyg X-1, this long timescale is not directly relevant to black-hole models for objects accreting at the Eddington limit. It has been suggested¹², however, that low-luminosity active galaxies are emitting at $\sim 10^{-3}$ of the Eddington limit; if so, these long timescales are exactly as predicted.

The EXOSAT results have improved X-ray timing observations of active galaxies to the point where realistic detailed models are necessary to interpret the data. In some ways this situation is similar to that of brownian motion before its explanation by Einstein. We now have high-quality time variability data with no obvious signatures. It is to be hoped that the ASTRO-C observations will greatly increase the database and determine

whether X-ray timing measurements can indeed constrain black-hole models of active galaxies. $\square$

1. Warwick, R. *Proceedings of the Meeting on Accretion onto Compact Objects 1987* (eds Mason, K. O., Watson, M. G. & White, N. E.) 195 (Springer, Berlin, 1987).
2. Lawrence, A., Watson, M. G., Pounds, K. A. & Elvis, M. *Nature* **325**, 694–696 (1987).
3. McHardy, I. & Czerny, B. *Nature* **325**, 696–698 (1987).
4. Fabian, A. *Proc. R. Soc. A* **366**, 499 (1979).
5. Lightman, A. in *X-ray Astronomy in the 1980s* (ed. Holt, S.) 142 (NASA TM 83848, 1982).
6. Meekins, J. F. *et al. Astrophys. J.* **278**, 288 (1984).
7. Nolan, P. *et al. Astrophys. J.* **246**, 494 (1981).
8. Lawrence, A. *Mon. Not. R. astr. Soc.* **192**, 83 (1980).
9. Mushotzky, R., Holt, S. S. & Serlemitsos, P. J. *Astrophys. J.* **255**, L115 (1985).
10. Tennant, A. & Mushotzky, R. F. *Astrophys. J.* **264**, 92 (1983).
11. Barr, P., Clavel, J., Giommi, P., Mushotzky, R. F. & Madejski, G. in *Proc. Conf. Variability Galactic and Extragalactic X-ray Sources* (ed. Treves, A.) (Como, Italy, 1986).
12. Barr, P. & Mushotzky, R. *Nature* **320**, 421 (1986).
13. Lawrence, A., Watson, M., Pounds, K. & Elvis, M. *Mon. Not. R. astr. Soc.* **217**, 685 (1985).
14. Urry, C. M. *et al. Proc. Conf. Variability Galactic and Extragalactic Sources* (ed. Treves, A.) (Como, Italy, 1986).
15. Mushotzky, R. *Adv. Space Res.* **13**, 157 (1984).

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Analytical geochemistry

Surfaces of reacted minerals

Harold C. Helgeson $\$37$

UNTIL recently, much of what we know, or think we know, about the mechanisms of interfacial reactions between silicates and aqueous solutions has been derived from subjective interpretation of indirect (and in many cases ambiguous) evidence, largely rate studies of mineral hydrolysis. Now, however, advanced technology is being used to examine and to analyse directly the surfaces of reacted mineral grains to determine the composition of their surface 'skins' and the extent to which they change with reaction progress. These data can be used to infer the stoichiometry of activated complexes at reactive surface sites and to elucidate the mechanisms of surface detachment during the dissolution of complex minerals such as feldspars, pyroxenes and amphiboles. Recent pioneering studies of this kind, one of which is reported by Jean-Claude Petit *et al.* on page 705 of this issue, use the specific resonant nuclear reaction technique to measure the total hydrogen concentration (H^+ , H_2O and H_3O^+) as a function of depth to about 1,000 Å from the outer surface of reacted diopside ($CaMg(SiO_3)_2$) grains at pH values of 2 and 6. The results of Petit *et al.* indicate that one or more of these species is present in high concentrations at depths of about 500 Å from the outer surface of the reacted diopside grains, and that the concentration is maximal at a depth of about 200 Å. Circumstantial evidence indicates that most of the hydrogen is present as

H_2O , but note that small concentrations of H_3O^+ relative to H_2O in the hydrogenated surface are sufficient to control the rate of mineral hydrolysis.

The steady-state pH dependence of mineral dissolution rates observed in the laboratory can be explained in terms of transition-state theory by assuming the presence of activated complexes involving hydrogen, hydronium or hydroxyl ions at the reactive sites. The stoichiometry of these activated complexes depends on the solution pH and the crystallochemical characteristics of the mineral. For example, recent theoretical consideration by W. M. Murphy (*Geochim. cosmochim. Acta*, in the press) of pyroxene, wollastonite and olivine dissolution rates reported in the literature indicates that the dissolution stoichiometries and pH dependence of these rates are consistent with steady-state rate control by transition-state decomposition of activated complexes with stoichiometries corresponding to $(H_2C(2))_{0.5}C(1)_{0.5}SiO_3^Z$, $(H_2Ca)SiO_3$ and $H(Mg,Fe)SiO_3^+$, respectively, where Z is the charge on the complex and C(1) and C(2) are the cations on the M(1) and M(2) octahedral sites, respectively. The resonant nuclear reaction technique and secondary ion mass spectrometry offer hope of direct confirmation of the existence of these species at surface reactive sites such as dislocation outcrops. But much remains to be done to discriminate between H^+ , H_3O^+ , H_2O , OH^- and other

species at particular sites. At the moment, only the total concentrations of elements can be measured, and even these are averaged over the surface of the mineral.

In addition to these two techniques, X-ray photoelectron spectroscopy is a powerful method of analysing the surfaces of reactant minerals. This technique has been used to demonstrate that Ca and Mg in the M(2) octahedral sites of dissolving diopside are preferentially released relative to SiO_2 from the outermost surface of the reacting mineral (Schott, J., Berner, R. A. & Sjöberg, E. L. *Geochim. cosmochim. Acta* **45**, 2123–2135; 1981, and Schott, J. & Berner, R. A. *Geochim. cosmochim. Acta* **47**, 2333–2340; 1983). Comparison of results using all three techniques indicates that the relation between penetration of hydrogen into the surface during dissolution and remobilization of the elements of the reactant mineral at the active surface sites is far more complex for crystalline silicates than for simple silicate glasses.

Studies of reacted mineral surfaces using the three methods I have mentioned above have important implications for many geochemical processes, including weathering, diagenesis and hydrothermal rock alteration. In addition, they should allow better understanding of reactions among ground waters and toxic waste materials, such as solid radioactive waste. Experimental techniques of this kind will help to elucidate the surface chemistry of silicates and hold great promise for unravelling the mechanisms of heterogeneous catalysis on mineral surfaces of both organic and inorganic reactions in geochemical processes. These include decarboxylation reactions and reduction of sulphate in natural waters.

Reactions among mineral surfaces and aqueous solutions is a complex process involving such phenomena as hydration of the surface, adsorption, ion exchange, complex formation, bond disruption and irreversible decomposition of activated complexes followed by surface detachment. Depending on the stoichiometric number per mole of energetically distinct crystallographic sites for cations present in a mineral, which ranges from three in pyroxenes such as diopside to nine in certain amphiboles, many surface species may be involved in the hydrolytic process. In particularly complex minerals such as amphiboles, dissolution mechanisms may involve parallel reactions rather than a simple series of elementary reaction steps. Only when technology is sophisticated enough to detect and unravel these complex mechanisms will we be able to claim with confidence that we can hope to understand the hydrolytic process. $\square$

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Vagaries of nomenclature

SIR—It is a pity that when zoological nomenclature is an Aunt Sally for non-taxonomists they should so often be uninformed or disingenuous. Seldom do they trouble to find a reason for a nomenclature requirement that seems to them arcane or futile. So it is with L.J. Bruce-Chwatt's remonstrance (*Nature* 325, 114; 1987) against the use of '-i' and '-ii' terminations for patronymic names of mosquito species. He argues that the '-ii' ending is 'wrong', because names such as *bancroftii* and *cruzii* honour Bancroft and Cruz, not Bancroftius and Cruzius, and therefore that by his lopping of the terminal '-i' when there are two he is using the 'grammatically correct spelling'.

This would be fine were it not for the fact that notional latinization of modern personal names (to Bancroftius, for example) before forming the genitives has been widespread and legitimate practice since the dawn of Latin-based nomenclature and is still permissible today. Would he have us 'correct' the multitude of such '-ii' names published since 1758 in every major animal group — or is he really after a dispensation for malariologists? Anyway, he should take heart. The use of '-ii' personal genitives for new species has been effectively dead for many years and is recommended against in the International Code of Zoological Nomenclature (Appendix D(III)16(b)).

Bruce-Chwatt fails to note the obvious risk inherent in what he has done, namely that those without his classical grounding (most of us), seeing that he reduces '-ii' to '-i', might assume that the second 'i' should always be lopped. He does not mention the point, but the names of several mosquitoes correctly end '-ii' because they are genitives based on personal names (usually Italian, Indian or Japanese) that happen to end with the letter 'i': *ficalbii*, *forattinii*, *goeldii*, *hatorii*, *martinii*, *onorii*, *purii*, are examples. There is also *fabricii* from the great (latinized) naturalist Fabricius and the geographical derived *mississippii*.

Under the present code, the correct spelling is the one published when the species was first described, whether or not the '-i' or '-ii' termination was used. This allows the correct spelling to be objectively determined by reference to that description — and obviates the need to trace all the subsequent history of the name in its scattered literature in an effort to discover who first altered it (or indeed whether it has ever been altered at all). Under the code all is clear; a variant subsequent spelling (one 'i' reduced to two or vice versa) is an 'incorrect subsequent spelling' (Article 33(d)) and cannot be validly used. The much-maligned and misunderstood code is right on this particular issue.

I cannot refrain from noting the paradox with which Bruce-Chwatt ends his letter. He blames the code for preserving stability, an unsettling experience for taxonomists used to obloquy for supposedly doing the opposite.

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Divergence and directional mutation pressures

SIR—Grivell¹ proposes that divergent genetic codes, as found in ciliated protozoa and *Mycoplasma* may be "relics of a primitive genetic code [that] possibly preceded the emergence of the so-called universal code". We suggest that a different explanation is more probable, and that such codes may be formed by capture of stop codons. Clues to this event in the case of ciliates are found by comparing the sequences of transfer RNAs.

Kuchino, Hanyu and co-workers^{2,3} found two 'new' glutamine tRNAs in *Tetrahymena thermophilus*, one with the anticodon UmUA (where Um is 2'-O-methyluridine), pairing with UAA and UAG, and the other with anticodon CUA, pairing with UAG. UAA and UAG are stop codons in the universal code, but in ciliated protozoa the only stop codon appears to be UGA. Differences in nucleotide sequences between the 'new' tRNAs and the other glutamine tRNA, with anticodon UmUG, in *Tetrahymena*, are 13 and 11 nucleotide substitutions in tRNA^{Gln}_{UmUG} when compared with tRNA^{Gln}_{UmUA} and tRNA^{Gln}_{CUA} respectively, and 4 substitutions in tRNA^{Gln}_{UmUA} compared with tRNA^{Gln}_{CUA} (ref. 3). These differences may be a rough clue as to time elapsed since evolutionary divergence⁴. Differences between yeast and rat or wheat tRNAs with the same anticodon are 13–15 substitutions⁵ and between *Drosophila* and vertebrates are 5–12 substitutions⁵. From this, one could conclude that the divergences of the tRNAs for Gln in *Tetrahymena* were well within the time of existence of eukaryotes.

Capture of stop codons could have taken place through a series of non-deleterious (neutral or adaptive) changes brought about by 'directional mutational pressure'⁶. Stop codons UAA and UAG have evidently disappeared from ciliates, perhaps during successive episodes of AT and GC pressure. AT pressure presumably now exists in ciliates, as shown by the low GC content (20–30 per cent) of their DNA. The gene for tRNA^{Gln}_{UmUG} duplicated and one of the duplicates, under AT pressure, acquired anticodon UmUA. Later, the tRNA gene with anticodon UmUA duplicated again, and in one of the duplicates, anticodon UmUA mutated to

CUA, which pairs strongly with UAG³.

During this period, some of the glutamine codons CAA and CAG mutated under AT pressure to UAA and UAG, pairing with the new glutamine anticodons UmUA and CUA. UAA and UAG have been identified as alternate glutamine codons in the ciliated protozoans *Tetrahymena*⁷, *Stylonichia*⁸ and *Paramecium*^{9,10}. This may be a result of recent stop codon capture¹¹. This is in contrast to the suggestion by Grivell¹ that "the ciliates branched off from the primitive eukaryotic ancestor very early in evolution, quite possibly at a time when protein translation was still tolerant of changes in the genetic code". No such tolerances need be postulated, for the observed changes are compatible with neutral substitutions, and account for the presence in *Tetrahymena*, of four glutamine codons, CAA, CAG, UAA and UAG, and three glutamine anticodons, UUG, UUA and CUA. An analogous pattern exists for leucine in the universal genetic code with codons CUA, CUG, UUA and UUG and anticodons, UAG, UAA and CAA.

Similarly, in *Mycoplasma capricolum* (25 per cent GC in DNA) AT pressure has resulted in the disappearance of UGA stop codons, replaced by UAA, and the mutation of anticodon CCA, in a duplicate of tryptophan tRNA, to UCA¹². Simultaneously, tryptophan codons UGG mutated to UGA. Anticodon UCA pairs with both UGG and UGA by 'wobble'. UGA has become a tryptophan codon, 'captured' by AT pressure^{12,13}.

We propose that *Tetrahymena* and *Mycoplasma* have both evolved to make translatable use of former stop codons¹⁴ and that the divergent code in ciliates may be of recent origin and derived from the eukaryotic code.

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1. Grivell, L.A. *Nature* 324, 109-110 (1986).
2. Kuchino, Y., Hanyu, N., Tashiro, F. & Nishimura, S. *Proc. natn. Acad. Sci. U.S.A.* 82, 4758-4762 (1985).
3. Hanyu, N., Kuchino, Y. & Nishimura, S. *EMBO J.* 5, 1307-1311 (1986).
4. Holmquist, R., Jukes, T.H. & Pangburn, S. *J. molec. Biol.* 78, 91-116 (1973).
5. Sprinzl, M., Vorderwiibecke, T. & Hartmann, T. *Nucleic Acid Res.* 13, r51-r104 (1985).
6. Sueoka, N. *Proc. natn. Acad. Sci. U.S.A.* 48, 582-592 (1962).
7. Horowitz, S. & Gorovsky, M.A. *Proc. natn. Acad. Sci. U.S.A.* 82, 2452-2455 (1985).
8. Helftenbein, E. *Nucleic Acids Res.* 13, 415-433 (1985).
9. Preer, J.R. Jr, Preer, L.B., Rudman, B.M. & Barnett, A.J. *Nature* 314, 188-190 (1985).
10. Caron, F. & Meyer, E. *Nature* 314, 185-188 (1985).
11. Lehman, N. thesis, Univ. California (1986).
12. Yamao, F. *et al. Proc. natn. Acad. Sci. U.S.A.* 82, 2306-2309 (1985).
13. Jukes, T.H. *J. molec. Evol.* 22, 361-362 (1985).

The pure and the applied

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£41

Biotechnology: The University-Industrial Complex. By Martin Kenney.
Yale University Press: 1986. Pp. 306. \$23.95, £22.50.

IN UNDER a decade, a whole new industry, capitalized with many billions of dollars, has come into being. Such is the rate of change in the late twentieth century. The product of 30 years of government support of basic biomedical research, biotechnology emerged from its incubator in the mid-1970s and quickly found its niche in the socio-economic structure of American and multi-national industry.

Mr Kenney chronicles, in intimate detail, the unfolding patterns of this development as determined by the extraordinary constraint that the basic knowledge — the blueprint for the industry — was almost exclusively to be found in the minds of university faculty. Industry had the production facilities and the marketing skills, but it was barren of the new expertise. The professorial start-up companies, the university-industry contracts, the limited partnerships in research and development, the equity purchases and licensing agreements by the multi-national corporations, all stemmed from this unprecedented circumstance.

Mr Kenney's book is repetitious and much of the material is redundant, but it does draw together a great deal of information about the establishment, financing and management of the new biotechnology companies. Also described are the subsequent contractual and fiscal interventions of the large chemical and pharmaceutical companies, as well as their successful efforts to purchase, in whole or in part, access to or even sole rights to the output of appropriate university departments, so as to acquire instant expertise and to ensure the economic future of the companies concerned.

Particular attention is paid to agricultural biotechnology, potentially the largest market. Organizational arrangements here were complicated because the land-grant universities, traditionally the source of agricultural research, have not been in the forefront of molecular biology, which is largely funded instead by the National Institutes of Health. Other universities, such as Harvard, MIT, Washington (St Louis) and Stanford, have consequently become the corporate partners for the development of plant molecular biology.

The effects on the academic world of these university-corporate relationships are examined, perhaps necessarily, in less satisfactory detail. Only sketchy anecdotal evidence is presented as to the impact

of corporate ties and proprietary information upon freedom of information in academia, upon the research and educational agendas, and upon the credibility of the universities as sources of disinterested judgement for society. In this regard, the author comments succinctly: "It is

Genentech makes splash on Wall Street

"One of the most spectacular market debuts in recent history." That was how the *Wall Street Journal* described the first day of public trading in shares in Genentech, the San Francisco company which has been among the leaders of those aggressively pursuing the commercial exploitation of recombinant DNA technology.

Initially the company had proposed to offer one million shares at between \$20 and \$30 each. But demand was so great that an initial price of \$35 was fixed for members of an underwriting syndicate through which the shares were made public last week. An extra 100,000 shares were made available, providing the company with an investment — once brokerage commissions had been deducted — of \$36 million.

During the day of frantic over-the-counter trading, in which more than half of the shares were resold by their original purchasers, the price rose at one point to \$89 a share, eventually dropping back to \$71. As the company has 7.5 million shares, this

Booming beginnings — a news story from Nature, October 1980.

quite remarkable how quickly doubts regarding safety receded once it appeared that profits could be made from this new technology". Similarly, Mr Kenney makes only limited reference to the questions of the propriety of the sequestration to private gain of knowledge gained at public expense, or the longer term consequences of "validating new areas of the natural world as private property" via the patent system.

The book is written from a capitalist-economic perspective, and does not cast a flattering light upon the molecular biology research community. Thus, on p.245 we find:

The biomedical funding system created an entire research structure based upon grantsmanship and the building of research empires... For the empires to keep functioning, products in the form of journal articles and prizes awarded are crucial. In this bitter competition research monies from any source are welcome because they could provide that competitive edge.

Mr Kenney is understanding, if not forgiving:

The professors who join companies have made rational choices, given the alternatives presented... Professors are also creatures of their social milieu. [This] is not to excuse the more egregious exploitation of students, the betrayal of the public trust, and even the theft that have occurred with creation of this industry.

He does not, however, relate these concerns to the responsibilities of universities to foster technology transfer — the transmission of the knowledge they have acquired with the aid of public funds to the industries wherein it may find useful application. Nor does he relate them to the increasing reliance, particularly in the United States, upon such high technology as the best means to maintain international competitiveness (which, in turn, affects the potential for university support). Such questions are particularly relevant to an industry by which, as the author states, "every production process that uses carbon-based materials" will be affected.

With the discovery that a previously arcane area of academic research suddenly had great market value, the traditional social relationships of the university became strained. Much of the recent development described here can be seen as an adaptation to that strain, but whether or not this is a transient adaptation remains to be seen.

It is only at the end of his book that Mr Kenney raises, suddenly and without answer, the most critical questions:

- The entire manner in which man conceptualizes interaction with the natural world will change as we begin to create new life forms — making "natural" selection a planned rather than a random process. Our world will be filled with genetically engineered organisms: if DNA is a "program" then these are "living robots". Ultimately we will need institutions to deal with the fact that we can engineer ourselves [p.246].

- Should the enormous power of transforming... life forms be transferred to groups merely seeking a return on investment [p.247]?

- To be fully serious, the debate must ask the questions of what can and cannot be privatized and done for profit. Industry cannot answer this question — the managers have a duty to maximize profit... This requirement for an unbiased debate raises the question of whether the universities and their professors are capable of carrying on this debate [p.246].

Biotechnology: The University-Industrial Complex bears upon questions of fundamental importance to science, academia and society, and provides valuable documentation of the magnitude of the actions already taken and the multitude of participants involved. But we must hope that someone will write the sequel, for which this is only the prologue. □

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What's going on in mathematics?

Thomas Banchoff *£28*

The Problems of Mathematics. By Ian Stewart. Oxford University Press:1987. Pp.257. Hbk £17.50, \$24.95; pbk £5.95, \$9.95.

FRACTALS, catastrophes and instantons — these are some of the strange objects that inhabit the current world of mathematics. We might see them once in a while mentioned in a newspaper article, quickly forgotten. We also hear that there are more mathematicians alive now than in all previous history combined. What is it that they all work on? What is the character of modern mathematical research and what is its relation to the familiar mathematics which is taught in schools? Is there any way for the lay person to begin to appreciate the answers to these questions?

Ian Stewart asks himself these things in an imaginary radio interview in the introduction to *The Problems of Mathematics*, and he concludes "Well, I suppose I could give it a try". He does in fact give it quite a good try. In 20 chapters, Dr Stewart surveys a great many of the areas of current research in mathematics, a formidable

task if one accepts the assertion that most practising mathematicians have difficulty communicating their ideas to mathematicians outside their field let alone the lay person.

As a mathematician myself, I appreciated reading introductions to several areas I had only vaguely heard about, especially since the author has done well in presenting the subjects I know best. As a geometer, however, I have to remark on the paucity of visual material in the book. In all there are only nine illustrations (one of which, the cycloid curve traced out by a point on a rolling wheel, is in error in two respects) and fourteen chapters have no pictures at all. I find it almost impossible to imagine discussion of the mathematics of knots which contains not a single picture of one, or a treatment of non-Euclidean geometry without even one triangle. On the other hand, there is also an absence of equations and complicated algebraic symbolism. To tell so much about modern developments using neither equations nor extensive diagrams is quite an accomplishment.

The chapters are generally independent, which makes for easier browsing, and Dr Stewart starts most of them with a discussion of a classical problem which has some present-day aspect. From Euclid's proof of the infinitude of the number of primes, we move to factorization problems used in 'unbreakable' codes. From the discovery of non-Euclidean geometries in the last century, we come to modern research on possible geometries of three-dimensional analogues of surfaces. From elementary probability, we progress to the mathematics of chaos. The historical context is handled quite well, except that the problems of 20 or 30 years ago are somewhat neglected compared to those of the present day. Thus the research work of each of the Fields medallists (the mathematical equivalent of Nobel laureates) of the past decade is discussed but there is little mention of those who went before. For those who wish to fill in the intervening background, Dr Stewart provides a reading list for each chapter at the end of the book.

This is an unusually fine collection of essays about modern research mathematics. The other volumes in Oxford's *The Problems of . . .* series claim to be directed towards sixth-formers or first-year university students. Although these might certainly profit from reading Dr Stewart's book, it is definitely suited to a much wider audience. Indeed, it should perhaps be required reading for professional mathematicians as well, so that we might all benefit from the author's success in interpreting our work to the larger world. □

Thomas Banchoff is a Professor of Mathematics at Brown University, Providence, Rhode Island 02912, USA.

One knock for "no"

Steve Blinkhorn *£30*

The Adventures of a Parapsychologist. By Susan Blackmore. Prometheus:1986. Pp.249. \$19.95.

PARAPSYCHOLOGISTS, like Roman Catholics, come in two principal varieties: the devout and the lapsed. Whilst the memoirs of the devout are at best improving and uplifting, those of the lapsed can have all the morbid fascination of the autobiography of a public hangman.

Dr Blackmore, however, her life's allotted span barely half complete, has devised a new genre. Here one can read about the opinions, adventures, paranormal experiences and half-hearted extramarital amour (in the singular) of a failed parapsychologist. Too slight to be a treatise, uncertain as a melodrama and written in the style of pulp romantic fiction, it is an account of a change of faith.

Experiencing her self out of her body, the author seeks to work for a PhD in extra-sensory perception (ESP), and is told she is out of her mind. With growing methodological rigour, she consistently fails to find any evidence to support her belief in the paranormal, and feels a failure: will she ever get to be a famous parapsychologist? Meanwhile her acquaintances in the field win fame, fortune and university lectureships. Perhaps, they say, she doesn't believe fervently enough — her sceptical tendencies are interfering with her *ganzfeld*.

For all that, she has chosen the better part. Whilst others degenerated into writing pap astrology for station bookstalls (snake oil being temporarily out of fashion), she gradually came to understand that ESP, telekinesis, Tarot card readings and the whole shabby collection of spurious contacts with a deeper reality that make up parapsychology are born of a failure to grapple with the cruel demands of decent scientific method. They represent the last throw of Cartesian dualism against the notion that people are ultimately biodegradable. So instead she renovated a cottage, tried to pull a psychokinetic stunt with a home pregnancy test and got a job in television presenting a socially-aware, pop-science series.

So why write a book about it? The nearest we get to a real parapsychological phenomenon is the distinct impression that Dr Blackmore has more prospects as a ghost writer than a ghost hunter. More than half of the chapters in the book run to exactly ten pages — surely a significant result beyond that predicted by chance? And when one gets a real sniff of something worth knowing about — such as the repeated suggestion that Carl Sargent's Cambridge experiments were slightly less

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pure than the driven ectoplasm — substantiating detail is curiously unforthcoming. Why was she told never to darken the doors of his laboratory again?

There are gloomy lessons here for psychologists in general. Why, when psychological phenomena abound, poorly explained but going to the marrow of the welfare both of people individually and of society as a whole, should anyone try to base a career on the investigation of effects that no one can even find? Why has the statistical significance of experimental effects come to be seen as more important than their size? How can anyone survive an undergraduate course in Psychology, Philosophy and Physiology (as the author did) without acquiring a glimmer of philosophical insight into the business of scientific inquiry?

In favour of the author it has to be said that (the demands of the ghost writer or editor notwithstanding) she does not

spare her own blushes over juvenilia, and one has the real sense that she stuck to method rather than yielding to peer group pressure. She found nothing worth reporting, and spends almost 250 pages telling us so.

Somewhere, waiting in the wings, are dewy-eyed undergraduates waiting to take up the torch of Rhine. Ouija boards at the ready, they will cling to their conviction that a mass of evidence for paranormal phenomena is unreasonably rejected by conventional scientists out of prejudice. Tarot card manufacturers have nothing to fear from this book. For whereas Dr Blackmore could have chosen to write a brave indictment, she has settled for self-indulgent apologia. □

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Highs and lows

George Fink *FL2*

Drugs and the Brain. By Solomon H. Snyder. W.H. Freeman: 1986. Pp.228. £15.95.*

SOLOMON Snyder's reason for entering medicine and medical research rather than becoming a student of philosophy was, as he states in his preface, that "philosophy is hardly a proper vocation for a nice Jewish boy". However, those who prefer the tight prose of Kurt Vonnegut may find Snyder's loquacious and at times awkward English a trifle heavy, and will be relieved that Snyder chose a career in medical research rather than in philosophy. Snyder probably entered a career in psychopharmacology because like many other boys, non-Jewish as well as Jewish (and not all "nice"), he found the study of the brain and the physical-chemical basis of the mind quite irresistible. This comes through clearly in *Drugs and the Brain*, which is by far the best book of its type that I have come across.

Using his formidable intellect, knowledge and resources, Snyder has produced a masterly work which explains clearly how drugs affect brain function. The general reader needs no more than an elementary knowledge of biology to understand the book, but at the same time *Drugs and the Brain* will delight the specialist. Snyder systematically covers all the psychotropic drugs — that is drugs which affect mood, thought, perception and behaviour — varying his approach

from chapters which focus on a class of drugs (opiates, for example) to those which focus on particular psychiatric disorders, such as schizophrenia, and the drugs which are used in their treatment.

Each chapter starts with a lively historical account which concentrates upon key personalities involved in the discovery, synthesis, application or marketing of the drug concerned. For example, as well as dealing with the well-known account of how cocaine was the undoing of Sigmund Freud, Snyder tells the fascinating tale of the origin of the Coca-Cola Company. Angelo Mariani in 1863 patented 'Vin Mariani' a wine containing coca extract which soon became one of the most popular drinks in Europe. For this, and for developing coca lozenges and tea (used for dyspepsia and sore throat), Mariani received commendations and a

special medal from Popes Pius IX and Leo XIII. John Pemberton, a pharmacist in Georgia, was inspired by Mariani's cocaine-containing preparations and in 1886 'designed' Coca-Cola as a stimulant and a treatment for headaches. Subsequently, Pemberton removed the alcohol from Coca-Cola, and the final formula for 'Classical Coke' was developed when, in 1888, Pemberton replaced ordinary water with soda water as the base. Asa Candler, another pharmacist bought the rights from Pemberton and founded the Coca-Cola Company in 1892. Cocaine was removed from the drink early in the twentieth century.

Snyder emphasizes that some drugs, although harmful in themselves, have formed the basis of drugs which have proved key armaments of modern medicine. Cocaine again serves as an excellent example. Its derivatives, Procaine, Lidocaine and Tetracaine, are potent local anaesthetics without which many modern surgical techniques could not easily be carried out. These anaesthetics have long been taken for granted and were originally synthesized from coca-leaves, but their success and popularity led drug companies to develop methods of synthesis which avoided the expense of importing coca-leaves. The 1980s scourge of cocaine might be partially alleviated and the livelihood of the Latin American peasants maintained if drug companies were persuaded to return to the older extraction methods which would require an almost complete diversion of all the coca-leaves from the synthesis of cocaine to the synthesis of local anaesthetics. Only a fool would believe that such a technical solution to a difficult social and economic problem is likely to be contemplated by politicians, let alone by the pharmaceutical companies and the major drug rings and their apparently respectable

IMAGE
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REASONS

Making a meal of it — the opening moves in phagocytosis of Paramecium by the larger protist Didinium nasutum. The picture is reproduced from Peter H. Raven and George B. Johnson's introductory textbook Biology, which will be among the books reviewed in Nature's annual textbook review issue on 12 March.

*In the United States published as a volume in the Scientific American Library.

dependents. However, Governments should at least be faced with the fact that there are several alternative equitable and moral solutions to the cocaine problem which do not necessarily require the destruction of peasant communities in the Andes.

The layout of the book is very attractive and some of the illustrations are strikingly beautiful. Classical coloured drawings of plants from which the various drugs are derived interleave with photographs of Inca ruins and reproductions of appropriate woodcuts, paintings and master drawings such as Albrecht Dürer's *Melen-colia*. No expense appears to have been spared to reproduce in colour histological and histochemically prepared sections of the brain, clear diagrams of the relevant neural pathways in the brain and schematic illustrations of how drugs act. Included are diagrams of the structure of the various drugs, their derivatives and, where appropriate, their endogenous counterparts. There are also portrait photographs of many of the key figures such as John Cade, the Australian psychiatrist who discovered the value of lithium for the treatment of mania, and Nathan Kline who discovered that iproniazide, a monoamine oxidase inhibitor that was first used in the treatment of tuberculosis, is a potent antidepressant. Real data are used judiciously to back up the simplified explanations in the text and figures.

If *Drugs and the Brain* has any fault it is that it entrenches the view that each neurotic and psychotic symptom is due to the malfunction of one specific chemical neurotransmitter system. However, this is the age of chemical phrenology, and so in a book written for the non-specialist it is indeed very difficult to paint a picture which is clear but which at the same time is sufficiently sceptical of the notion that each mood, feeling, perception or behaviour is mediated by one specific neurotransmitter.

Solomon Snyder has produced a book which will be enjoyed by anyone with an interest in one of the most dramatic and fascinating advances in biology, the way neurons communicate to produce the properties of what we call the mind and how these communications are altered by drugs. *Drugs and the Brain* should be read and understood by politicians, civil servants and members of committees responsible for dealing with the enormous social and economic problems caused by drugs that affect the brain. The specialist too will wish to purchase the book. Not only will it look very fetching on the coffee-table now, but it could well become a collector's item in 25 years time. □

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How well fed is the world?

Dennis T. Avery *£32*

People, Food, and Resources. By Kenneth Blaxter. Cambridge University Press: 1986. Pp. 118. £15, \$29.95.

Technology in the 1990s: Agriculture and Food. Edited by Kenneth Blaxter and Leslie Fowden. The Royal Society: 1986. Pp. 179. Distributed by Cambridge University Press, £32.50, \$54.50.

In *People, Food, and Resources* Sir Kenneth Blaxter makes a serious, well-intentioned effort to assess the dangers of rising world population and limited food supply. He concludes that the outlook is poor because continued high population growth is certain and increased food production is not. For all the good intentions, however, both Sir Kenneth and the contributors to the Royal Society volume which he co-edited leave their readers struggling in the Club of Rome's ignorance *circa* 1974 — the report of that date predicted that famine was soon to sweep the world. The books do not reveal that in the 1980s the world's food production is expanding even faster than its fast-growing population and may still be picking up speed.

According to the statistics of the UN Food and Agriculture Organization (never known for minimizing the problem of hunger in the world) per capita food production in the developing countries gained 17 per cent in the period 1980 to 1984 alone. The developing countries raised their farm output by 38 per cent during the decade 1974–1984, and 4.1 per cent annually in the 1980s. This is a much more rapid increase than the historic 2.5 per cent per year cited by Sir Kenneth. Virtually all of the recent increase in farm output in less-developed countries came from higher yields rather than extending plantings onto fragile acres or cutting down forests. This is an astounding human achievement, one which readers will not winnow out from Sir Kenneth's determined pessimism and bias towards population management.

Sir Kenneth can be forgiven part of his sour outlook; the population growth curve has long been the least tractable element of the food–population equation. But most population experts now concede that the curve is S-shaped rather than L-shaped. As the developing nations become more affluent, their population growth will slow. Sir Kenneth says in *People, Food, and Resources* that he sees no reason to expect this to occur — but most of us know that in modern societies additional children cease to add wealth or social insurance. They become instead a

major net expense to the family.

In his most interesting chapter, Sir Kenneth offers a nice tribute to the much-maligned Reverend Malthus. Then he proceeds to follow Malthus into the trap of assuming that population will always out-run food supply — and there is now far more reason to believe in the potential fruits of agricultural research than there was in 1800.

To Sir Kenneth's credit, he apparently made a legitimate effort to find out the prospects for increasing world food production. But his specialists failed him. *Technology in the 1990s: Agriculture and Food*, the report of The Royal Society's conference on food production, lacks perspective, is highly uneven in quality and the issues concerned are obscured by over-technical language. Take this, for example: "Peptides corresponding to the antigenic site of food-and-mouth disease virus which have been synthesized either by the solid-phase method or as a part of the fusion protein have elicited neutralizing antibody and protect cattle against challenge infection . . .". The passage hardly informs the lay reader that the first, fully safe vaccine for foot-and-mouth disease has now been produced through genetic engineering.

We should not be surprised, however. Researchers have not made a good job of public liaison in the dozen years since the Club of Rome report. They should have been telling us about the powerful new techniques in plant genetics and the farming systems that are transforming agriculture in the less-developed countries, and about the potential of new lines of research. Perhaps they feared overstatement, or believed that a rush of public concern would be good for their budgets. Their lack of communication — and our resulting ignorance — has been expensive for everybody. Farmers in the affluent countries have been misled about the value of their land and the need for their produce. Consumers and taxpayers throughout the OECD countries have paid dearly to finance farm surpluses the Third World doesn't need. And few of the less-developed countries themselves have understood their farming opportunities clearly. These two books won't do much to help any of them. □

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● Just published by W.W. Norton is the fourth of the *State of the World* reports from the Worldwatch Institute. Like its predecessors, the 1987 edition "examines the counterpoint of urgency and uncertainty that has come to dominate world affairs in an age when the environmental consequences of human activities transcend national boundaries"; included are chapters on, among other topics, world agriculture, nuclear power, re-cycling of waste materials and the chemical cycles. Price is hbk \$18.95, pbk \$9.95.

The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing

Tom Maniatis & Robin Reed

\$480

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A small set of distinctive short RNA molecules are found in the nuclei of all higher eukaryotic cells and yeast, in protein complexes known as 'small nuclear ribonucleoprotein particles', or snRNPs. Recent work has confirmed early suggestions that these particles form part of the machinery by which primary RNA transcripts are processed to their mature, functional form. In particular, snRNPs have been shown to be an integral part of the 'spliceosome', a multi-component complex involved in the removal of intron sequences from the coding regions of messenger RNA precursors.

THE precise excision of intervening sequences during RNA splicing is an interesting example of the high degree of specificity involved in biosynthetic processes. Self-splicing RNA precursors achieve this specificity primarily through intramolecular interactions¹ whereas all other types of RNA splicing require interactions between cellular factors and specific recognition signals in the RNA precursors. The primary candidates for the cellular recognition factors involved in splicing of mRNA precursors (pre-mRNA) are the small nuclear ribonucleoprotein particles (snRNPs). This notion was initially based on the observation that sequences at the 5' end of the RNA of one Sn RNP, U1 RNA, are complementary to the consensus 5' splice site²⁻⁴. Although experimental evidence implicating snRNPs in pre-mRNA splicing has accumulated during the past five years, definitive proof was obtained only recently.

Much of the recent progress in elucidating the function of mammalian snRNPs has resulted from the development of efficient *in vitro* splicing systems⁵⁻⁷. Both normal and mutant RNA precursors can be synthesized in large amounts using plasmids containing the bacteriophage SP6 promoter^{8,9}, and these precursors are accurately spliced in nuclear extracts⁷. Interactions between snRNPs and pre-mRNA have been studied during the splicing reaction using a variety of approaches including the use of antibodies directed towards either the protein components of snRNPs¹⁰ or the trimethylguanosine cap of the snRNAs^{11,12}. In addition, targeted nuclease digestion of specific snRNAs and the purification of splicing complexes have provided new insights into the function of snRNPs.

Substantial progress has also been made recently in the analysis of yeast pre-mRNA splicing and in the characterization of yeast snRNPs. The purpose of this review is to summarize the evidence showing that snRNPs play an essential role in mammalian and yeast pre-mRNA splicing, and to discuss recent advances in understanding the interactions between snRNPs and pre-mRNA.

Mammalian splicing and snRNPs

The snRNPs are ribonucleoprotein particles found in the nuclei of all higher eukaryotic cells (see refs 13 and 14 for reviews). snRNPs consist of one snRNA molecule, ranging in size from 56 to 217 nucleotides, and a set of approximately 10 different proteins. Some of these proteins are unique to a particular snRNP whereas others are common to all particles. The major species, U1, U2, U4, U5 and U6 are nucleoplasmic, are present at an abundance of 200,000 to 1,000,000 copies per cell, and contain a trimethylguanosine cap (with the exception of U6 snRNA which contains an unidentified cap structure). In addition, these snRNPs are associated with the Sm antigen, which is recognized by sera from patients with autoimmune disease¹⁰. Most snRNPs are isolated as monomeric particles, but U4 and U6 are recovered from cells as a single entity^{15,16}. A fraction of

the U1 and U2 snRNPs also appear to be complexed with each other¹⁷. Recently, four new snRNPs were discovered. Designated U7¹⁸, and U8-U10¹⁹, they were not previously detected because of their relatively low abundance. It is possible that additional snRNAs are present in mammalian cells but have eluded detection.

The first experimental evidence that snRNPs are involved in splicing was provided by antibody inhibition studies. Anti-snRNP antibodies were shown to inhibit splicing in isolated nuclei²⁰, and subsequently in splicing extracts^{21,22}. Unfortunately, these studies did not provide unequivocal evidence for a role of snRNPs in splicing because the addition of partially purified snRNPs to antibody-depleted extracts did not restore splicing activity²².

A more direct demonstration that snRNPs play a role in splicing was provided by experiments in which the RNA component of snRNPs was inactivated by nuclease digestion. Treatment of splicing extracts with micrococcal nuclease, followed by EGTA inactivation of the nuclease, completely inhibits RNA splicing. In contrast to the antibody-inhibition studies, splicing activity could be restored to nuclease-treated extracts by the addition of partially purified snRNPs which are themselves devoid of splicing activity²³. To demonstrate involvement of individual snRNP types in the splicing reaction, targeted nuclease digestion was used to inactivate specific snRNAs. This was accomplished by hybridizing synthetic DNA oligonucleotides to specific sequences in snRNAs and then treating the DNA-RNA hybrid with the enzyme ribonuclease (RNase) H which degrades the RNA strand of the RNA-DNA duplexes²². This approach was used initially to show that RNase H cleavage of the 5' end of U1 snRNA inhibits splicing²²⁻²⁴. Similar RNase H cleavage experiments were subsequently employed to demonstrate a requirement for U2 (refs 23, 24), U4 (refs 25, 26) and U6 (refs 26) snRNPs in the splicing reaction. Splicing activity could be reconstituted by combining extracts in which different snRNAs were inactivated²³⁻²⁶. Thus, the inhibitory effect of RNase H cleavage could be directly linked to the inactivation of specific snRNAs.

Progress in understanding the mechanism of pre-mRNA splicing led to more-specific questions regarding the role of snRNPs in the splicing reaction. Studies *in vitro* revealed that splicing proceeds by a two-step mechanism (refs 7, 27-29; see ref. 30 for review). The first step entails cleavage at the 5' splice site and the formation of a 2'-5' phosphodiester bond between the 5-terminal G residue of the intron and an A residue located ~30 nucleotides upstream from the 3' splice site. The second step involves cleavage at the 3' splice site, ligation of the two exons, and release of the excised intron as a lariat. Sequence comparisons^{28,31,32} and functional studies³³⁻³⁵ indicated that, in addition to the conserved 5' and 3' splice junction sequences, the site of lariat formation is a recognition element for pre-

mRNA splicing. Indeed, the branchpoint sequence is protected from oligonucleotide-directed RNase H cleavage and RNase A digestion during the splicing reaction³⁶. This protection is not observed in the absence of ATP or with precursors containing splicing mutations. A possible function for snRNPs in branchpoint protection was suggested by the observation that binding to the branchpoint sequence is not detected in extracts treated with micrococcal nuclease³⁶.

Specific interactions

The first demonstration that snRNPs can interact specifically with pre-mRNA was provided by experiments showing that extensively purified U1 snRNP binds to the 5' splice site of an *in vitro* synthesized mouse β -globin pre-mRNA³⁷. Significantly, snRNP proteins are required for binding specificity. In more recent experiments, purified U1 snRNP was shown to bind specifically to 5' splice-site sequences in RNA³⁸. Surprisingly, specific binding was also observed with single-stranded DNA containing 5' splice-site sequences. A low level of 3' splice-site activity was observed with U1 snRNP, but the functional significance of this interaction, if any, is not known.

When *in vitro* splicing systems were developed, interactions between specific snRNPs and pre-mRNA could be analysed during the splicing reaction. This was accomplished using an RNase protection and immunoprecipitation assay in which labelled pre-mRNA is added to an *in vitro* splicing extract, incubated under splicing conditions, and digested with RNase T1 in the presence of anti-snRNP antibodies^{12,24}. Regions of the pre-mRNA that interact specifically with snRNPs are protected from nuclease digestion and precipitated by the anti-snRNP antibodies. This approach was used to show that U1 snRNP binds to the 5' splice site and that U2 snRNP binds to the branchpoint sequence²⁴. These splicing recognition sequences are not protected from RNase T1 cleavage if the snRNPs are inactivated by oligonucleotide-directed RNase H cleavage. An additional snRNP, possibly U5, interacts specifically with the 3' splice site¹². The functional significance of the specific interactions between snRNPs and pre-mRNA was demonstrated by showing that RNase protection is not observed with pre-mRNAs containing mutations in the splicing recognition elements^{12,24}.

The biochemical requirements for the binding of different snRNP types to their recognition sequences vary. Specific binding to the 5' and 3' splice sites is observed immediately after the pre-mRNA is added to the extract at 0°C, and ATP is not required^{12,24}. In contrast, binding to the branchpoint sequence requires incubation at 30°C in the presence of ATP^{12,24,36}. These observations suggest that the first event in the splicing reaction is binding to the 5' and 3' splice sites. Binding to the branchpoint sequence then occurs as the assembly of the splicing complex proceeds.

Genetic evidence for base pairing

As discussed above, three different snRNPs have been implicated in site-specific binding to pre-mRNAs (U1, U2, and U5). The observed binding specificities may involve base-pairing interactions between the snRNA and pre-mRNA, such as that proposed for U1 snRNA²⁻⁴, and/or snRNP proteins may mediate specific binding to pre-mRNA. Although models for base-pairing between U2 snRNA and pre-mRNA have been proposed^{39,40}, the suggested complementarity is less striking than that observed with U1 snRNA. Finally, examination of the U5 snRNA sequence failed to reveal significant complementarity with the 3' splice-site consensus sequence¹².

The first evidence for base-pairing interactions between U1⁴¹ and U2⁴² snRNPs and high-molecular-mass nuclear RNA was provided by *in vivo* psoralen cross-linking studies. Because specific binding to splicing recognition sequences was not demonstrated, these studies did not provide an experimental link between base-pairing and splicing. More recently, this link was demonstrated by a direct genetic test⁴³. The experiments

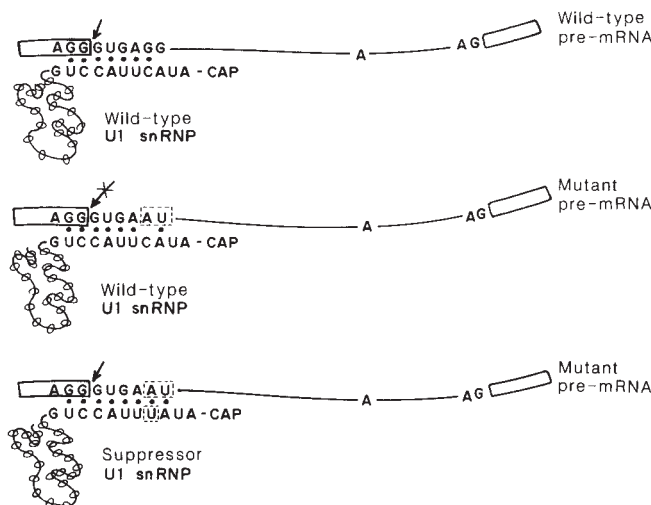


Fig. 1 The suppression of a mutation in the adenovirus *Ela* 5' splice site by a compensatory base change in U1 snRNA. The pre-mRNA is represented by the open boxes (exons) and thin lines (introns). The *Ela* 12S 5' splice site, branchpoint sequence and 3' splice site are indicated by GUGAGG, A and AG, respectively. Wild-type U1 snRNA is capable of forming 7 contiguous base pairs with the 5' splice site. In the mutant pre-mRNA, one of these base pairs is disrupted by the G-to-A change at position 5. Changing the C at position 4 of U1 snRNA to a U results in the re-establishment of base pairing at position 5 of the mutant pre-mRNA, and suppression of the splicing mutation⁴³. The arrow indicates the site of cleavage at the 5' exon-intron junction and the base changes in mutant pre-mRNA and the suppressor U1 snRNA are indicated by the open boxes with the dashed lines.

were designed to determine whether point mutations in the 12S and 13S 5' splice sites of the adenovirus *Ela* gene, which disrupt potential base-pair interactions, can be suppressed by compensatory base changes in human U1 snRNA. Plasmids containing the mutant *Ela* genes and either the wild-type or suppressor mutants of the U1 gene were transfected into HeLa cells. Analysis of RNA obtained from the transfected cells revealed that a mutation at position +5 in the 12S splice site was efficiently suppressed by U1 RNA containing the compensatory base change. The potential base-pair interactions between the wild-type and mutant 12S 5' splice sites and U1 snRNA are illustrated in Fig. 1. Interestingly, increasing the complementarity between the wild-type 12S 5' splice site and U1 snRNA by introducing a single base change into U1 RNA led to more efficient use of the 12S splice site in the wild-type *Ela* RNA precursor. Thus, one of the important determinants in 5' splice site recognition appears to be the extent of complementarity between U1 snRNA and the 5' splice site. Although these studies indicate the base-pairing between the 5' splice site and U1 snRNA is necessary for splicing, this interaction alone is not sufficient since suppression could not be achieved with a splicing mutation at +3 in the 12S splice site⁴³. The possibility that protein-RNA interactions are involved in specific snRNP binding to pre-mRNA is suggested by the identification of an snRNP protein that binds specifically to the 3' splice-site sequence⁴⁴⁻⁴⁶.

Splice-site selection

Correct splice-site selection requires discrimination between bona fide splice sites and the many sequences in RNA precursors that closely resemble or are identical to consensus 5' and 3' splice sites (pseudo-splice sites). These pseudo-sites are never used in the presence of the normal splice sites, but at least some of them (cryptic splice sites) are used efficiently when the normal sites have been inactivated by mutation^{47,48}. These observations indicate that the consensus splice-site sequences are necessary but not sufficient for accurate splicing. The second aspect of

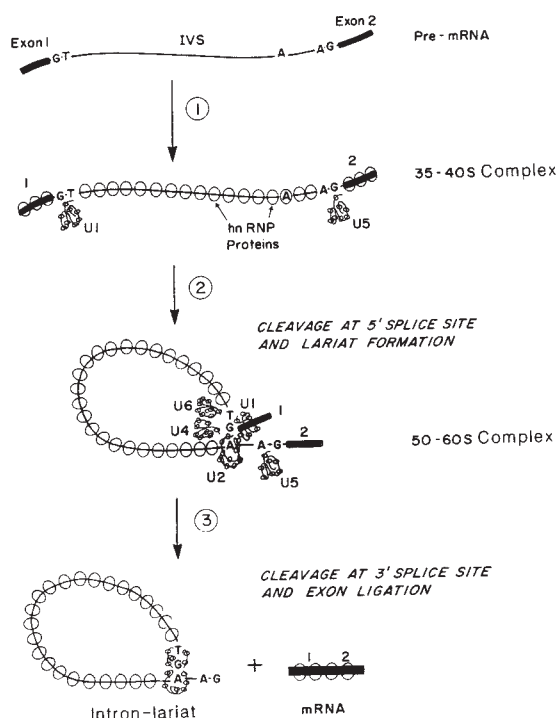


Fig. 2 A model for spliceosome assembly. In the first step a 35–40S complex is formed in which U1 and U5 snRNPs bind to the 5' and 3' splice sites respectively, and hnRNP proteins bind at unspecified locations. In the second step, U5 snRNP binds to the 3' splice site and cleavage at the 5' splice site and lariat formation occurs. The site at which the U4 and U6 snRNP complex binds is not known. The complex thus formed sediments at 50–60S. In the final step, cleavage at the 3' splice and exon ligation leads to the release of the intron as a lariat containing factors bound to the branchpoint sequence.

splice-site selection involves the mechanism by which the appropriate pairs of 5' and 3' splice sites are chosen in mRNA precursors containing multiple introns. Although the splice sites in different introns of the same precursor are nearly identical, pairing of a 5' splice site in one intron with a 3' splice site in another is rarely observed in normal pre-mRNAs. Thus, the splice-site sequences alone are not sufficient to specify correct splice-site pairing in multi-intron RNA precursors.

Based on the demonstration of specific interactions between snRNPs and splice-site recognition sequences described above, snRNPs are likely to be involved in both aspects of splice-site selection. Considering that the splicing recognition sequences alone are not sufficient to specify correct splice-site selection, other sequences must be involved. Progress in identifying these sequences was recently provided by the analysis of precursors containing duplicated 5' or 3' splice sites⁴⁹. These experiments revealed that deletions, substitutions or even subtle changes of exon sequences can affect the use of adjacent splice sites. In many cases these changes result in the complete inactivation of the splice site adjacent to the altered exon. The nature of the sequences within the normal flanking exons required for the efficient use of the adjacent splice site is not known. Higher-order structure of the exons may facilitate binding of snRNPs or other splicing factors to splice sites. Alternatively, the normal exon sequences may be required for optimal splicing complex formation and stability.

Experiments with the splice-site duplication precursors may also be relevant to the mechanism of correct pairing of 5' and 3' splice sites in precursors containing multiple introns. In particular, the data indicate that when duplicated splice sites are flanked by identical sequences, the pair of 5' and 3' splice sites in closest proximity to one another are preferentially used⁴⁹.

Spliceosomes and snRNPs

Understanding the detailed mechanisms of pre-mRNA splicing will require the identification and purification of individual splicing components. However, progress in this direction has been hampered by the complex nature of the splicing apparatus. Several factors other than snRNPs, including heterogeneous nuclear RNP (hnRNP) proteins⁵⁰, appear to be required for the splicing reaction^{23,51–53}. Preliminary fractionation of splicing components by column chromatography was made possible by the development of *in vitro* complementation assays^{23,51–53}. However, difficulties in recovering sufficient quantities of active splicing factors have impeded progress using this approach.

An alternative approach for isolating splicing components is to identify factors that specifically interact with the 5' splice site, 3' splice site, or the branchpoint sequence^{44–46}. Two factors identified in this way may mediate interactions between snRNPs and their recognition sequences in pre-mRNA. One factor enhances the binding of U1 snRNP to the 5' splice site⁴⁴. Another factor, which binds specifically to the 3' splice site, can be immunoprecipitated by anti-Sm monoclonal antibodies^{45,46}. Interestingly, the binding activity co-fractionates with U5 snRNP⁴⁵, which is consistent with the proposal that this snRNP binds to the 3' splice site¹². This factor can be dissociated from snRNPs with high concentrations of Mg^{2+} , but the dissociated factor retains splice-site binding activity⁴⁵. Thus, the specific binding of U5 snRNP to pre-mRNA may be mediated by protein-RNA interactions, rather than by base-pairing.

Splicing components have also been identified using techniques designed to purify the entire splicing complex, designated the spliceosome^{54–56}. Spliceosomes were originally detected by adding labelled RNA precursors to splicing extracts, incubating under conditions that are optimal for splicing, and then sedimenting the reaction mix through a sucrose or glycerol density gradient^{54–58}. Although considerable variability has been reported in the sedimentation coefficients of the complexes and the kinetics of their formation, it is generally agreed that two classes of complexes can be observed (Fig. 2). One complex, which sediments at 35–40S, forms at 0 °C in the absence of ATP and its formation does not require functional 5' and 3' splice sites in the pre-mRNA. A 35–40S complex is also the first particle observed at 30 °C in the presence of ATP. As the splicing reaction proceeds, a second complex is observed at 50–60S. Its formation requires ATP, incubation at 30 °C and normal 5' and 3' splice sites. Depletion of hnRNP proteins from the splicing extract⁴⁷ or inactivation of snRNPs^{55,56,58} prevents the formation of this complex. Finally, both unspliced pre-mRNA and splicing intermediates are found in the gradient fractions containing this complex. The 50–60S complex is missing one or more essential components, since incubation of the sucrose gradient fractions in splicing buffer does not produce additional splicing products. However, when splicing extract is mixed with the complex, splicing products are generated and the reaction is accelerated⁵⁶.

A hypothetical pathway for spliceosome assembly is shown in Fig. 2. This pathway, which is based on the sequence and factor requirements for spliceosome assembly *in vitro* and on RNase protection experiments, provides a useful working model, but may or may not reflect the actual steps involved in spliceosome assembly *in vivo*. For example, because a precursor-product relationship has not been established, the 35–40S complex could be an *in vitro* dead-end complex rather than a bona fide precursor.

Purification of intact spliceosomes has been difficult because they appear to aggregate and associate with non-specific proteins and RNA in the nuclear extract. In an attempt to circumvent these problems, the polyanion heparin was added to the splicing reaction after spliceosome formation^{59,60}. However, this treatment decreases the sedimentation coefficient of the spliceosome from 60S to 35S, suggesting that specific as well as non-specific components may have been dissociated. These 'core' complexes have been characterized by both gel electrophoresis⁵⁹ and affinity

chromatography⁶⁰. In the latter approach, a small number of biotinylated nucleotides were incorporated into an RNA precursor. This precursor was then incubated in a splicing extract, treated with heparin, fractionated on a glycerol gradient, and finally, purified by selection on streptavidin agarose beads. Analysis of the small RNAs associated with the purified 35S core complexes revealed the presence of U2, U5, U4 plus U6 snRNAs, and an unidentified RNA species, X. The conspicuous absence of U1 snRNA from the complex could be a result of the heparin treatment, or U1 snRNP may be bound to the splicing complex only transiently⁶⁰. Significantly, quantitative analysis of the snRNAs associated with the complex indicated that each snRNP is present at roughly one copy per pre-mRNA.

Although the characterization of spliceosomes has provided interesting insights into the nature of the components associated with the splicing complex, the preparative purification of intact, functional spliceosomes has yet to be achieved. Conditions must be found which prevent aggregation and non-specific binding to nuclear components, but do not inactivate the spliceosome. On the basis of the information obtained thus far, the spliceosome appears to be a dynamic structure assembled on the pre-mRNA template, possibly in a stepwise fashion, and then dissociated as the splicing reaction is completed. Purification of a homogeneous population of splicing complexes may require blocking the splicing reaction at different steps. An important point to emphasize is that the components involved in spliceosome assembly are freely exchangeable since extracts in which different splicing factors have been inactivated efficiently complement each other *in vitro*. Like the ribosome, the spliceosome appears to be composed of relatively stable subunits (the snRNP particles), and many factors that are involved in the assembly of an active complex on the RNA substrate. Purification of functional splicing complexes may therefore prove to be a significant challenge.

Yeast pre-mRNA splicing

Advances in understanding yeast pre-mRNA splicing have paralleled those achieved in mammalian systems. Both *in vivo*⁶¹⁻⁶³ and *in vitro*^{63,64} studies with the yeast *Saccharomyces cerevisiae* have shown that the two-step splicing mechanism established in mammalian systems is also used in the processing of yeast nuclear pre-mRNAs. In addition, a number of studies have demonstrated the importance of the 5' and 3' splice sites and the branchpoint sequence in yeast pre-mRNA splicing⁶⁵⁻⁷². Finally, preliminary biochemical fractionation studies have begun to identify splicing factors⁷³, and density gradient sedimentation has shown that splicing occurs in a 40S spliceosome⁵⁴.

In spite of many similarities between yeast and metazoan splicing, a number of differences have been noted. First, the yeast 5' splice site and branchpoint sequences are highly conserved although the corresponding sequences in metazoan pre-mRNAs vary significantly⁶⁵⁻⁶⁷. Second, mutations in the yeast splice sites and the branchpoint sequence usually inactivate splicing⁶⁵⁻⁶⁷ whereas the corresponding mutations in mammalian introns usually lead to the use of cryptic sites^{33,35,48,49}. Third, unlike metazoan pre-mRNAs, most yeast 3' splice sites do not contain an extensive polypyrimidine tract^{69,72}, and other evidence suggests that the polypyrimidine sequences may not be essential for cleavage at the 5' splice site and lariat formation *in vitro*⁷². In contrast, the first step in splicing does not occur when the 3' splice site is deleted in mammalian introns^{33,55,74}. Although mammalian mRNA precursors are not spliced in *Saccharomyces cerevisiae*^{75,76}, yeast pre-mRNA is accurately spliced in mammalian cell extracts. However, the lariat forms at a different site⁷⁷. Taken together, these observations suggest that the sequence elements at the branchpoint and the 3' splice site may have somewhat different functions in yeast and metazoan splicing.

The differences in sequence requirements for splicing in yeast

and metazoa may be a consequence of the way genes are organized and expressed in the two types of organisms. Although a few genes in *S. cerevisiae* contain one or two small introns, most are intron-free. In contrast, the majority of higher eukaryotic genes contain multiple introns, which range in size from 30 to several thousand nucleotides. A number of higher eukaryotic genes also encode transcripts that are differentially spliced³⁰. The mechanisms required to accurately remove multiple introns from a single RNA precursor without exon deletion, and to regulate cell-specific differential splicing, may have necessitated greater flexibility in the sequence requirements for RNA splicing in higher eukaryotes.

Until recently, functional homologue of mammalian snRNPS had not been identified in yeast. Yeast snRNPS could not be immunoprecipitated with anti-Sm antibodies² nor detected by cross-hybridization with clones snRNA genes⁷⁸⁻⁸². However, at least two different yeast snRNAs contain binding sites for the Sm antigen, since these snRNAs can be immunoprecipitated with human anti-Sm antibodies after the snRNAs are injected into *Xenopus* oocytes⁸³. Thus, the specific interaction between the Sm-antigen and yeast snRNAs has been conserved, but the yeast Sm-antigen is not recognized by antibodies raised against the mammalian analogue. Although yeast cells contain at least 24 different snRNAs with a tri-methyl cap at their 5' termini⁸², these RNAs differ significantly from their presumed metazoan counterparts. Yeast snRNAs are encoded by single-copy genes^{79,80}, are present at only 10-500 copies per cell⁷⁸ and range in size from 120 up to 1,000 nucleotides⁸². Attempts to investigate the function of yeast snRNAs, and possibly establish a link to pre-mRNA splicing, proved difficult because gene disruption techniques revealed that many of the snRNA genes are not essential for growth⁷⁹⁻⁸¹. This observation suggested that the gene products are either functionally redundant or that they are not essential for splicing.

The development of a yeast *in vitro* splicing system^{61,63-64} provided the opportunity to investigate the involvement of snRNPs in pre-mRNA splicing. A function for snRNPs was first suggested by the observation that yeast splicing extracts are inactivated by treatment with either micrococcal nuclease or trimethyl-cap antibodies⁷³. In subsequent experiments, the association of specific snRNAs with the splicing complex was demonstrated by analysis of spliceosomes purified by gel electrophoresis or affinity chromatography⁸⁴. When yeast spliceosomes were treated with high concentrations of salt, EDTA and large amounts of nonspecific carrier RNA to disrupt nonspecific RNA-protein interactions, three distinct complexes were separated by gel electrophoresis⁸⁵. Analysis of the snRNA molecules associated with the three complexes revealed that the first complex formed is associated with an snRNA greater than 1000 nucleotides in length, designated large spliceosomal RNA1 (LSR1)⁸⁵ or snR20⁸². The formation of this complex precedes cleavage at the 5' splice site and lariat formation. The next complex formed contains three different small snRNAs designated snR7S, snR7L and snR14⁸², in addition to LSR1. The final complex contains LSR1, snR7S, snR7L, but is missing snR14. Both the splicing intermediates and products are found in the final complex, suggesting that the release of snR14 precedes cleavage at the 3' junction and exon ligation. Although this putative pathway of yeast spliceosome assembly provides a useful working model, it is important to emphasize that the spliceosome was treated with high salt, EDTA and nonspecific competitor RNA before gel electrophoresis. Therefore, the electrophoretic behaviour of the splicing complexes and the differences in the associated snRNAs may reflect the conditions used to analyse the complexes. Moreover, there is no direct evidence for a precursor-product relationship between the different complexes.

Recently, a surprising relationship between the 1,000-nucleotide yeast snRNA LSR1 and mammalian snRNPs was serendipitously discovered⁸⁶. In a negative control for U2 transcrip-

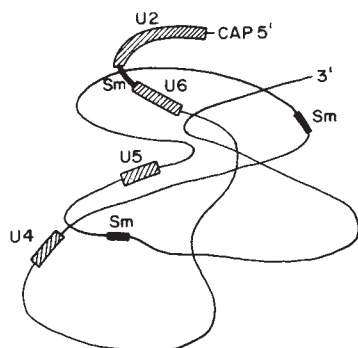


Fig. 3 The organization of the yeast LSR1 snRNA. Hatched boxes, regions homologous to sequences that are highly conserved in different mammalian snRNAs; thick black lines, possible binding sites for the Sm antigen⁸⁶.

tion experiments, an oligonucleotide complementary to U2 snRNA was used to prime cDNA synthesis with total yeast RNA. Unexpectedly, a cDNA was synthesized, and its sequence was found to be nearly identical to human U2 snRNA. An RNA larger than 1,000 nucleotides in length was detected when the human U2 oligonucleotide was used to probe a Northern blot of yeast RNA. Human U2 snRNA is only 189 nucleotides in length. When the gene encoding the large yeast RNA was cloned and sequenced, not only was extensive homology to human U2 snRNA detected, but short stretches of sequences were observed that are highly conserved in metazoan U4, U5 and U6 snRNAs (Fig. 3). Although these observations raise the interesting possibility that LSR1 is a 'poly snRNP' equivalent to a complex of U2, U5 and U4/U6 snRNPs in metazoans⁸⁷, the regions of homology are very short, and they are not found in secondary structures that are highly conserved in metazoan snRNAs. Elucidating the function of the unusually large LSR1 snRNA must await the analysis of point mutations in the conserved regions, and the characterization of other snRNAs associated with the yeast spliceosome.

The association of LSR1 with the spliceosome and its sequence homology to mammalian snRNAs, strongly suggest that it is involved in splicing. Further evidence was provided by gene disruption experiments showing that the LSR1 gene is essential for yeast viability⁸⁶. An interesting aside is that LSR1 is present in *Saccharomyces cerevisiae*, but not in *Schizosaccharomyces pombe*⁸⁶, where the human U2 hybridization probe detects an RNA that is approximately the same size as human U2 RNA. This observation may correlate with the finding that metazoan pre-mRNAs are not spliced in *Saccharomyces cerevisiae*, but at least one mammalian pre-mRNA is spliced in *Schizosaccharomyces pombe*⁸⁷. However, since different mammalian pre-mRNAs were tested in the two species, a generalization at this point may be premature. In any case, a number of differences between *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been noted, including the role of *ras* proteins, cell cycle control and transcriptional regulation. In all of these examples, *Schizosaccharomyces pombe* appears to be more closely related to metazoa than *Saccharomyces cerevisiae*⁸⁸.

All the elements necessary for a concerted biochemical and genetic analysis of yeast pre-mRNA splicing are now in place. In addition to identifying and characterizing the genes encoding the snRNAs involved in splicing, a number of other genes required for splicing have been identified. A class of temperature-sensitive mutants designated *rna*^{89,90} were shown to be defective in nuclear pre-mRNA splicing^{91,92}. Definitive evidence that at least three of the *rna* gene products are directly involved in pre-mRNA splicing was recently provided by *in vitro* complementation experiments⁹³. In these studies, splicing extracts prepared from mutant cell lines were shown to be temperature sensitive (ts) for splicing. These extracts can be complemented by heat-treated extracts prepared from different ts mutants⁹³.

Thus, it is only a matter of time before many of the genes essential for yeast pre-mRNA splicing are cloned and characterized, and their products produced in amounts suitable for detailed biochemical studies.

The role of snRNPs in 3' end processing

The snRNPs have also been implicated in other types of RNA processing. For example, specific interactions between snRNPs and pre-mRNAs play a role in 3' end formation of histone mRNAs (see ref. 94 for review). In this case, U7 snRNA base-pairs with a 3' processing signal located within the histone mRNA precursor⁹⁵. The functional significance of the base-pair interactions was recently demonstrated by showing that mutations in the 3' recognition element could be suppressed by compensatory base changes in U7 snRNA⁹⁵.

There may also be a function of snRNPs in 3' end processing of polyadenylated mRNAs. This possibility is supported by the observations that accurate *in vitro* polyadenylation is inhibited by anti-Sm and anti-U1 antibodies⁹⁶, and that the AAUAAA sequence required for polyadenylation associates with a factor that is precipitated by anti-Sm or anti-trimethyl cap antibodies⁹⁷. However, oligonucleotide-targeted RNase H digestion of U1, U2, U4 snRNAs alone or in combination has no effect on *in vitro* cleavage or polyadenylation^{25,97}. Even more puzzling is the observation that micrococcal nuclease treatment of nuclear extracts prevents 3' end formation^{97,98}, but the activity can be restored by the addition of purified RNA from a variety of sources⁹⁸. Thus, the *in vitro* polyadenylation reaction appears to have a general requirement for RNA mass, and possibly an snRNP other than U1, U2 or U4. At present, the antibody-inhibition and nuclease-digestion data cannot be reconciled.

Possible mechanisms of snRNP action

The mechanism by which specific interactions between snRNPs and pre-mRNAs lead to site-specific cleavage or ligation is not known, but a number of interesting possibilities exist. First, snRNPs may be 'ribozymes', by analogy with RNase P where the RNA component of a ribonucleoprotein complex is able to cleave transfer RNA precursors at a specific site⁹⁹. Another analogy is the ribosome: recent studies have shown that a binary complex of 16S and 23S ribosomal RNA is capable of catalysing many of the steps of protein synthesis in the presence of a limited number of ribosomal proteins¹⁰⁰. This observation raises the interesting possibility that the catalytic function of ribosomes is provided by ribosomal RNA whereas ribosomal proteins merely serve to finely tune the structure and activity of the RNA^{1,101}. Second, snRNPs may be a part of a scaffold structure that holds the appropriate regions of the pre-mRNA in close proximity so that the splicing event occurs without the involvement of protein enzymatic activity. This is suggested by the observation that class II yeast mitochondrial mRNA precursors are self-splicing, and the mechanism involves a lariat intermediate^{102,103}. Thus, both types of pre-mRNA appear to be spliced by the same mechanism, but one requires snRNPs and the other does not. The function of snRNPs in nuclear pre-mRNA splicing may therefore be served by intramolecular interactions in self-splicing class II mitochondrial pre-mRNAs. Third, snRNPs may be trafficking particles that carry the appropriate enzymes to their specific sites of action. For example, endonucleases and ligases may be bound to snRNPs. Although these alternatives cannot be distinguished now, the stage is set for determining the precise role of snRNP particles in mammalian and yeast pre-mRNA splicing.

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1. Cech, T. R. & Bass, B. L. *A. Rev. Biochem.* **55**, 599 (1986).
2. Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. *Nature* **283**, 220 (1980).
3. Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1877 (1980).
4. Mount, S. M. & Steitz, J. A. *Nucleic Acids Res.* **9**, 6351 (1981).
5. Padgett, R. A., Hardy, S. F. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5230 (1983).

6. Hernandez, N. & Keller, W. *Cell* **35**, 89 (1983).
7. Krainer, A. R., Maniatis, T., Ruskin, B. & Green, M. R. *Cell* **36**, 993 (1984).
8. Green, M. R., Maniatis, T. M. & Melton, D. A. *Cell* **32**, 681 (1983).
9. Melton, D. A. *et al. Nucleic Acids Res.* **12**, 7035 (1984).
10. Lerner, M. R. & Steitz, J. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5495 (1979).
11. Lührmann, R. *et al. Nucleic Acids Res.* **10**, 7103 (1982).
12. Chabot, B., Black, D. L., LeMaster, D. M. & Steitz, J. A. *Science* **230**, 1344 (1985).
13. Reddy, R. & Busch, H. *Prog. Nucleic Acid Res. molec. Biol.* **30**, 127 (1983).
14. Brunel, C., Sri-Widada, J. & Jeanteur, Ph. *Prog. molec. subcell. biol.* **9**, 1 (1985).
15. Bringmann, P. *et al. EMBO J.* **3**, 1357 (1984).
16. Hashimoto, C. & Steitz, J. A. *Nucleic Acids Res.* **12**, 3283 (1984).
17. Mattaj, I. W., Habets, W. J. & VanVenrooij, W. J. *EMBO J.* **5**, 997 (1986).
18. Galli, G., Hofstetter, H., Stunnenberg, H. G. & Birnstiel, M. L. *Cell* **34**, 823 (1983).
19. Reddy, R., Henning, D. & Busch, H. *J. biol. Chem.* **260**, 6088 (1985).
20. Yang, V. W., Lerner, M. R., Steitz, J. A. & Flint, S. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1371 (1981).
21. Padgett, R. A., Mount, S. M., Steitz, J. A. & Sharp, P. A. *Cell* **35**, 101 (1983).
22. Kramer, A., Keller, W., Appel, B. & Lührmann, R. *Cell* **38**, 299 (1984).
23. Krainer, A. R. & Maniatis, T. *Cell* **42**, 725 (1985).
24. Black, D. L., Chabot, B. & Steitz, J. A. *Cell* **42**, 737 (1985).
25. Berget, S. M. & Roberson, B. L. *Cell* **46**, 691 (1986).
26. Black, D. L. & Steitz, J. A. *Cell* **46**, 697 (1986).
27. Grabowski, P. J., Padgett, R. A. & Sharp, P. A. *Cell* **37**, 415 (1984).
28. Ruskin, B., Krainer, A. R., Maniatis, T. & Green, M. R. *Cell* **38**, 317 (1984).
29. Padgett, R. A., Konarska, M. M., Grabowski, P. J., Hardy, S. F. & Sharp, P. A. *Science* **225**, 898 (1984).
30. Padgett, R. A., Grabowski, P. J., Konarska, M. M., Seiler, S. & Sharp, P. A. *Rev. Biochem.* **55**, 119 (1986).
31. Zeitlin, S. & Efstratiadis, A. *Cell* **39**, 589 (1984).
32. Keller, E. B. & Noon, W. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7417 (1984).
33. Reed, R. & Maniatis, T. *Cell* **41**, 95 (1985).
34. Rautman, G. & Breathnach, R. *Nature* **315**, 430 (1985).
35. Ruskin, B., Greene, J. M. & Green, M. R. *Cell* **41**, 833 (1985).
36. Ruskin, B. & Green, M. R. *Cell* **43**, 131 (1985).
37. Mount, S. M., Pettersson, M., Hinterberger, A., Karmas, A. & Steitz, J. A. *Cell* **33**, 509 (1983).
38. Tatei, K. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6281 (1984).
39. Ohshima, Y., Itoh, M., Okada, N. & Miyata, T. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4471 (1981).
40. Keller, E. B. & Noon, W. A. *Nucleic Acids Res.* **13**, 4971 (1985).
41. Calvet, J. P. & Pederson, T. *Cell* **26**, 363 (1981).
42. Calvet, J. P., Myer, L. M. & Pederson, T. *Science* **217**, 456 (1982).
43. Zhuang, Y. & Weiner, A. M. *Cell* **46**, 827 (1986).
44. Mayeda, A., Tatei, K., Kitayama, H., Takemura, K. & Ohshima, Y. *Nucleic Acids Res.* **14**, 3045 (1986).
45. Tazi, J. *et al. Cell* **47**, 755 (1986).
46. Gerke, V. & Steitz, J. A. *Cell* **47**, 973 (1986).
47. Wieringa, B., Meyer, F., Reiser, J. & Weissmann, C. *Nature* **301**, 38 (1983).
48. Treisman, R., Orkin, S. & Maniatis, T. *Nature* **302**, 591 (1983).
49. Reed, R. & Maniatis, T. *Cell* **46**, 681 (1986).
50. Choi, Y. D., Grabowski, P. J., Sharp, P. J. & Dreyfuss, G. *Science* **231**, 1534 (1986).
51. Furneaux, H. M., Perkins, K. K., Freyer, G. A., Arenas, J. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4351 (1985).
52. Kramer, A. & Keller, W. *EMBO J.* **4**, 3571 (1985).
53. Perkins, K. K., Furneaux, H. M. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 887 (1986).
54. Brody, E. & Abelson, J. *Science* **228**, 963 (1985).
55. Frendeway, D. & Keller, W. *Cell* **42**, 355 (1985).
56. Grabowski, P. J., Seiler, S. R. & Sharp, P. A. *Cell* **42**, 345 (1985).
57. Kaltwasser, E., Spitzer, S. G. & Goldenberg, C. J. *Nucleic Acids Res.* **14**, 3687 (1986).
58. Bindereif, A. & Green, M. R. *Molec. cell. Biol.* **6**, 2582 (1986).
59. Konarska, M. M. & Sharp, P. A. *Cell* **46**, 845 (1986).
60. Grabowski, P. J. & Sharp, P. A. *Science* **233**, 1294 (1986).
61. Domdey, H. *et al. Cell* **39**, 611 (1984).
62. Rodriguez, J. R., Pikielny, C. W. & Rosbash, M. *Cell* **39**, 603 (1984).
63. Newman, A. J., Lin, R. J. Cheng, S. C. & Abelson, J. *Cell* **42**, 335-344 (1985).
64. Lin, R. J., Newman, A. J., Cheng, S. C. & Abelson, J. *J. biol. Chem.* **260**, 14780 (1985).
65. Langford, C. J. & Gallwitz, D. *Cell* **33**, 519 (1983).
66. Pikielny, C. W., Teem, J. L. & Rosbash, M. *Cell* **34**, 395 (1983).
67. Langford, C. J., Klinz, F. J., Donath, C. & Gallwitz, D. *Cell* **36**, 645 (1984).
68. Parker, R. & Guthrie, C. *Cell* **41**, 107 (1985).
69. Cellini, A., Felder, D. & Rossi, J. J. *EMBO J.* **5**, 1023 (1986).
70. Cellini, A., Parker, R., McMahon, J., Guthrie, C. & Rossi, J. *Molec. cell Biol.* **6**, 1571 (1986).
71. Vijayraghavan, U. *et al. EMBO J.* **5**, 1683 (1986).
72. Rymond, B. C. & Rosbash, M. *Nature* **317**, 735 (1985).
73. Cheng, S. C. & Abelson, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2387 (1986).
74. Ruskin, B. & Green, M. *Nature* **317**, 732.
75. Langford, C., Mellen, W., Niessing, J. & Gallwitz, D. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1496 (1983).
76. Watts, F., Castle, C. & Beggs, J. *EMBO J.* **2**, 2085 (1983).
77. Ruskin, B., Pikielny, C. W., Rosbash, M. & Green, M. R. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2022 (1986).
78. Wise, J. A. *et al. Cell* **35**, 743 (1983).
79. Tollervey, D., Wise, J. A. & Guthrie, C. *Cell* **35**, 753 (1983).
80. Tollervey, D. & Guthrie, C. *EMBO J.* **4**, 3873 (1985).
81. Guthrie, C., Riedel, N., Parker, R., Serdlow, H. & Patterson, B. in *Yeast Cell Biology* (ed. Hicks, J.) 301-322 (Liss, New York, 1986).
82. Riedel, N., Wise, J. A., Serdlow, H., Mak, A. & Guthrie, C. *Proc. natn. Acad. Sci. U.S.A.* **21**, 8097 (1986).
83. Riedel, N., Wolin, S. & Guthrie, C. *Science* **235**, 328 (1987).
84. Pikielny, C. W. & Rosbash, M. *Cell* **45**, 869 (1986).
85. Pikielny, C. W., Rymond, B. C. & Rosbash, M. *Nature* **324**, 341-345 (1986).
86. Ares, M. Jr *Cell* **47**, 49 (1986).
87. Kaufer, N., Simanis, V. & Nurse, P. *Nature* **318**, 78 (1985).
88. Russell, P. & Nurse, P. *Cell* **45**, 781 (1986).
89. Hartwell, L. H. *J. Bact.* **93**, 1662 (1967).
90. Hartwell, L. H., McLaughlin, C. S. & Warner, J. R. *Molec. gen. Genet.* **109**, 42 (1970).
91. Rosbash, M., Harris, P. K. W., Woolford, J. L. & Teem, J. L. *Cell* **39**, 603 (1981).
92. Fried, H. M., Pearson, J. J., Kim, C. H. & Warner, J. R. *J. biol. Chem.* **256**, 10176 (1981).
93. Lustig, A. J., Lin, R. J. & Abelson, J. *Cell* **47**, 953 (1986).
94. Birnstiel, M. L., Busslinger, M. & Strub, K. *Cell* **41**, 349 (1985).
95. Schauffle, F., Gilmartin, G. M., Bannworth, W. & Birnstiel, M. L. *Nature* **323**, 777 (1986).
96. Moore, C. L. & Sharp, P. A. *Cell* **41**, 845 (1985).
97. Hashimoto, C. & Steitz, J. A. *Cell* **45**, 581 (1986).
98. Ryner, L. C. & Manley, J. L. *Molec. cell. Biol.* **7**, 495 (1987).
99. Altman, S. *Cell* **36**, 237 (1984).
100. Burma, D. P., Tewari, D. S. & Srivastava, A. K. *Arch. Biochem. Biophys.* **239**, 427 (1985).
101. Noller, F. H. & C. R. *Science* **212**, 403 (1981).
102. Peebles, C. L., Mecklenburg, K. L., Perlman, P. S., Tabor, J. & Cheng, H. L. *Cell* **44**, 213 (1986).
103. Van der Veen, R. *et al. Cell* **44**, 225 (1986).

ARTICLES

Topography of the core-mantle boundary and lateral homogeneity of the liquid core

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Separate inversions of travel-time residuals of waves reflected from and transmitted through the core-mantle boundary yield similar results in terms of spherical harmonic expansion of its topography up to degree and order 4. This indicates absence of detectable lateral heterogeneity in the liquid core. The relief of the boundary is ± 6 km but its flattening, determined by the c_2^0 term, does not depart significantly from the hydrostatic theory.

It is now possible to address the question of aspherical structure of the Earth over the range of depths from its centre to the surface. This is being accomplished using data that include waveforms of body and mantle waves, travel times of seismic pulses and decomposition of the fine structure of spectral peaks of free vibrations of the Earth. Information contained in these data sets is not independent, and the results of the interpretation can be verified by comparing the derived models. For example, Giardini *et al.*¹ in their Figs 3 and 4 compare models of the lower mantle obtained using three very different sources of data:

splitting of normal modes¹, waveform inversion of body wave and mantle wave data² and travel time residuals³ (see the cover of this issue). The agreement between models derived from different kinds of data stops above the core-mantle boundary (CMB), except that the available information indicates the presence of lateral heterogeneity below that level^{1,3-9}.

The CMB is the most dramatic discontinuity in the Earth's internal structure, concerning physical and chemical properties as well as the timescale of the processes that take place on either side of it. Retrieval of its shape is important not only for our

being able to address, for example, the question of lateral heterogeneity in the inner core, but also for our understanding of the geodynamic processes of the mantle or the magnetic field generation in the outer core.

The CMB represents only one element in the Earth's structure. The ultimate goal is to combine all the available data and invert them for the aspherical structure of the entire Earth. However, because of the difference in size of the subsets of data and their sensitivity to different elements of the Earth's structure, it is possible in such a procedure to introduce undesirable trade-offs and obscure an otherwise clear picture. At the present stage when the modelling strategy is still developing it is desirable to isolate an individual region within the Earth, use the available information on the structure above it and select the data that are particularly sensitive to its properties.

Assuming that the large scale mantle structure is known with sufficient accuracy we perform an experiment that focuses primarily on the shape of the CMB. In the process of verification of the result, using an independent set of data, we must, however, address a different question: that of the presence of lateral heterogeneity in the outer core.

The approach

The datum most sensitive to the depth of a discontinuity is the travel time of a wave reflected from its surface. For the reflection from the top of a discontinuity at a radius r , the perturbation in travel time, δt , due to a change in its radius, δr , is

$$\delta t = -\frac{2\delta r}{r} (\eta^2 - p^2)^{1/2} \quad (1)$$

where $\eta = r/v(r)$ and p is the ray parameter¹⁰. In principle, if the velocity $v(r)$ is known, an observation of the travel-time anomaly of a reflected phase can be immediately converted into an equivalent change of radius of a discontinuity.

Phases reflected from the CMB have designations PcP and ScS for the compressional and shear waves, respectively. In routine reporting procedures, the arrival times of PcP are more numerous and reliable because of the sharper onset of this phase, owing to lower attenuation.

With the known mantle structure, a well distributed set of observations of PcP travel times would be sufficient to retrieve the shape of the CMB. For a spherically symmetric Earth model this was the approach that led Jeffreys¹¹ to his estimate of the core radius of $3,473.1 \pm 2.5$ km; not far off from the currently used value of $3,480$ km¹². However, the data may not be uniformly distributed, and (in addition) there are possible sources of systematic errors that might put any such result in serious doubt, rendering the result inconclusive. Standard analysis, based on the assumption that the scatter in the data is random and uncorrelated, is meaningless if the sources of systematic errors, such as earthquake location or mantle structure, cannot be quantified and removed.

In addition to phases reflected from the CMB, there are waves transmitted through it: these are the so called core phases. For the purpose of a study based on routine reports, the most important phases are PKP (the AB and BC branches) and PKIKP (the DF branch). The latter designation denotes waves that traversed the inner core, and because there are reasons to suspect that this region is also laterally heterogeneous, we shall not use PKIKP in this experiment. Figure 1 shows the ray path of PcP and PKP phases and the inset is a plot of travel times of the core phases.

For waves transmitted through a discontinuity, the expression for perturbation in travel time is

$$\delta t = -\frac{\delta r}{r} [(\eta_+^2 - p^2)^{1/2} - (\eta_-^2 - p^2)^{1/2}] \quad (2)$$

where the subscripts + and - denote, respectively, the values of η on the top- and bottom-side of the discontinuity¹⁰. The

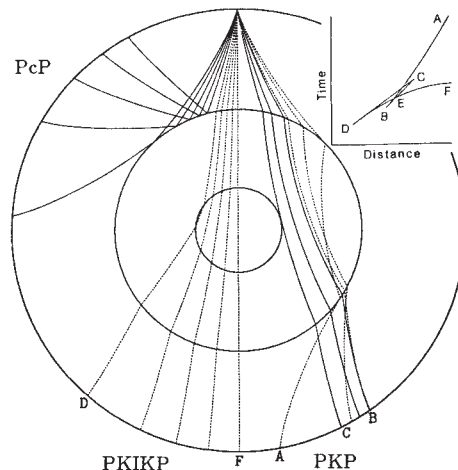


Fig. 1 Ray paths of waves reflected from core-mantle boundary, PcP, core phases bottoming in the outer core (branches BC and AB, broken line) and reaching the inner core: PKIKP (DF branch). Inset: diagram showing the travel times of the AB, BC and DF branches.

correction (2) must be considered for both the entry and exit points.

The velocity across the CMB changes abruptly: it drops from 13.7 km s^{-1} to 8 km s^{-1} ; this makes the travel times of core phases relatively sensitive to the CMB radius. Considering a particularly simple case of vertical incidence ($p=0$), a change in travel time of PcP is -0.146 s for a 1 km increase in the core radius; it is $+0.051 \text{ s}$ for PKP, but this must be counted twice, so a spherically symmetric increase in radius by 1 km would add 0.102 s to the travel time. Not much less than for PcP, but what is particularly important, the sign is opposite.

Adopting the approach of Dziewonski¹³ of expanding the lateral heterogeneity in spherical harmonics, we write

$$\delta r(\theta, \phi) = \sum_{l=0}^L \sum_{m=0}^l [c_l^m \cos m\phi + s_l^m \sin m\phi] p_l^m(\cos \theta) \quad (3)$$

where θ and ϕ are co-latitude and longitude and p_l^m are associated Legendre polynomials.

This may be substituted into equation (1) for PcP with the coordinates (θ, ϕ_r) corresponding to those of the reflection point. Such a system of equations of conditions can then be solved for the unknown coefficients c_l^m and s_l^m ; L is the cutoff value determined empirically depending on the quantity, quality and distribution of the data. For PKP the procedure is similar, other than the equations of condition contain contributions from both the entry and exit points.

The experiment

The International Seismological Centre (ISC), an organization involving representatives from 40 countries, is compiling arrival times of seismic phases and other parameters that are contributed by over 1,000 globally distributed stations. These individual reports are associated with seismic events (earthquakes, explosions) and are published in monthly bulletins (BISC)¹⁴. Since 1964 the Bulletin information has also been available on magnetic tapes, which makes it feasible to retrieve and process a very large amount of data. The first such use of BISC, in the context of studying lateral heterogeneity of the Earth, was reported in 1975¹⁵ and the use of this database is rapidly proliferating^{3,4,6-8,13,16-20}. It is important to investigate the signal-to-noise ratio of this data set, because it may have a bearing on how models using this database should be parameterized.

The data for P, PcP, and PKP (BC branch) are extracted from the years from 1964 to 1982 for earthquakes that have at least 50 P-wave arrivals in the teleseismic distance range (25° - 100°)

and not less than 5 arrivals in at least three azimuthal quadrants. The earthquakes are relocated using the most current travel-time table¹³ (only events shallower than 50 km are used), azimuth independent station corrections²¹ and corrections for ellipticity¹⁰. Data with an absolute value of a residual greater than 4 s are rejected. We define 'source regions' as having a constant area corresponding to $5^\circ \times 5^\circ$ squares at the Equator and 'receiver regions'. All travel-time residuals for a particular phase connecting the same source-receiver pair are averaged to yield 'summary residuals'. The data set for P is by far the largest: 1,549,235 individual readings are averaged into 66,569 summary residuals; for PcP there were 26,125 individual readings combined into 5,182 summary data and for PKP_{BC} there were 82,403 observations combined into 4,160 summary residuals.

Depending on the seismic activity of a particular source region and the density of stations in a receiver region there is substantial variability in the number of individual observations, N , used to derive a summary datum. We sort all summary data into subsets according to N . We define the variance σ_N^2 as:

$$\sigma_N^2 = \frac{1}{M_N} \sum_{i=1}^{M_N} (\delta t_i^{(N)} - \bar{\delta t}^{(N)})^2 \quad (4)$$

where M_N is the number of source receiver pairs with the count of N observations, $\delta t_i^{(N)}$ are the summary residuals and $\bar{\delta t}^{(N)}$ is the average residual of the N th population.

Figure 2 shows the plots of σ_N^2 as a function of N for the phases: P *a*, PcP *b* and PKP_{BC} *c*. If all residuals were due to random errors, we would expect $\sigma_N^2 = \sigma_E^2/N$. On the other hand, if all residuals were caused by a deterministic process, such as the large wavelength lateral heterogeneity, we should have $\sigma_N^2 = \sigma_H^2 = \text{constant}$, as there is no reason to expect that the level of lateral heterogeneity and number of connections for various source-receiver pairs are correlated. The results shown in Fig. 2 demonstrate that the actual σ_N^2 is a composite of the two models proposed above:

$$\tilde{\sigma}_N^2 = \sigma_E^2/N + \sigma_H^2 \quad (5)$$

Using linear regression we can estimate σ_E^2 and σ_H^2 ; the numerical results are shown in the figure. For the most numerous P-wave data set the r.m.s. residual of $(\sigma_N^2 - \tilde{\sigma}_N^2)$ is only 0.025 s^2 . The results illustrate that the BISC data set is noisy, with the variance, due to the effects that can be modelled deterministically, an order of magnitude smaller from that due to random noise (reading errors, lateral heterogeneity on a scale smaller than our $5^\circ \times 5^\circ$ division).

The conclusion from this analysis is that the BISC database contains signals related to lateral heterogeneity but, because of the low signal-to-noise ratio, the model parameterization should be chosen such as to assure substantial spatial averaging which would lead to the improvement of the ratio. Otherwise, it is easy to obtain a model that is dominated by noise. The P-wave data set described above was inverted to yield model HELM46²², similar to L02.56¹³ but derived using a larger set of observations; both models are defined by 245 parameters. This can be compared with nearly 50,000 model parameters determined by Clayton and Comer¹⁷, who used a similar data set. Because of the very uneven sampling of different elements of the Earth's volume by the BISC database, the optimum parameterization should consider a variable spatial resolution. Such a model has yet to be derived.

The data for PcP and PKP extracted from BISC according to the procedure described above are corrected for the effect of lateral heterogeneity predicted by model HELM46. Figure 3a shows the distribution of the reflection points of our PcP data set. It is nonuniform, with the coverage being poor in the Southern Hemisphere. For this reason we limit the expansion to degree and order 4; the coverage should be sufficient to determine perturbations with a resolving half-wavelength of 45° .

The map of the CMB obtained from inversion of the PcP data is shown in Fig. 3b. The largest feature is an area centred

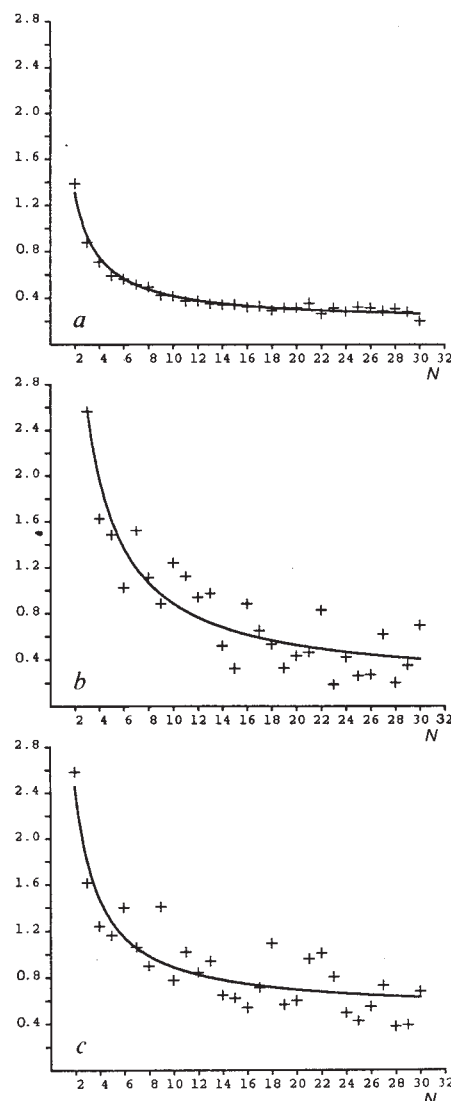


Fig. 2 Average variance of 'summary residuals' as a function of a number of individual observations, N , from which they were derived. *a*, P-waves; *b*, PcP and *c*, PKP_{BC}. Results of linear regression in search for σ_E^2 and σ_H^2 are shown for the individual data sets. In *a*, $\tilde{\sigma}^2(N) = 2.0/N + 0.23$; *b*, $\tilde{\sigma}^2(N) = 7.2/N + 0.16$; *c*, $\tilde{\sigma}^2(N) = 3.9/N + 0.50$.

on Azores, elevated above the average by slightly more than 5 km; another high extends over the eastern Pacific; three lows, of the order of -4 km , are seen south of Japan and Colombia and north-east of New Zealand. The standard error analysis indicates that these variations are statistically significant. This, however, does not consider the possible errors introduced by mislocation of earthquakes or incomplete accounting for the mantle effects. The rim of the Pacific is depressed; this is a region of high velocities in model L02.56 of Dziewonski¹³ (see map at a depth of 2,500 km). This would be consistent with the inference of Hager *et al.*²³, except for the features in the Pacific and the fact that the c_2^0 term is nearly zero.

The variance reduction of 3.5% appears misleadingly small. With the starting variance of 2.62 s^2 , however, this corresponds to 0.092 s^2 , or 57% of the total variance σ_H^2 which can be deterministically modelled (see Fig. 2b).

By itself the result, while interesting, might be considered inconclusive. Fortunately, there are other data (core phases) with resolving properties distinctly different from those of PcP. As Fig. 1a shows, the pattern of arrival times of core phases is relatively complex, particularly in the distance range from 144°

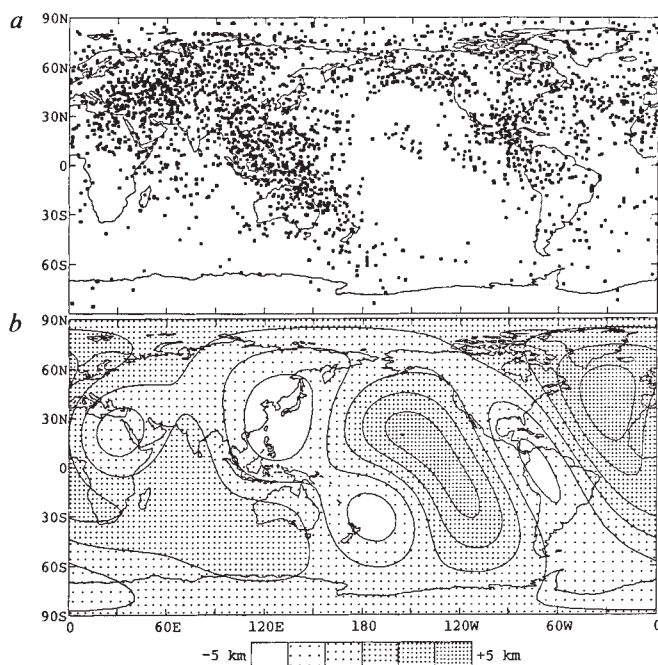


Fig. 3 *a*, Location of points of PcP reflection of 'summary data' used in this study. *b*, Topography of core-mantle boundary obtained by inversion of PcP residuals, corrected for lower mantle heterogeneity, for spherical harmonic coefficients up to degree and order 4.

to 150°–152°, where three branches, AB, BC and DF, are very close to each other. This picture, however, is misleading because it does not contain, in an explicit form, the amplitude information.

The retrograde AB branch has low amplitudes, is non-minimum-time and the signals are the Hilbert transforms of the original pulse, it is reported in BISC relatively infrequently and the data show large scatter. In addition, at distances beyond 160° the tunnelling effect of the mantle P-wave, nearly grazing the CMB, cannot be ignored²⁴. On the other hand, the BC branch is relatively short (144°–154°), is prograde, has large $|d^2t/d\Delta^2|$ and, therefore, large amplitudes and, in effect, is frequently reported. The complication is that the BC branch is very close to the PKIKP (DF branch) between 145° and 151°. It was pointed out, however, by Andersson and Cleary²⁵, that 85% of arrivals reported in the BISC (for this distance range) belong to the BC branch (see their Fig. 6), with the BC arrivals between 144° and 148° having amplitudes several times greater than DF pulses. Their finding is fully confirmed by our analysis, and we proceed on the assumption that all arrivals centred about the theoretical BC travel-time curve within ± 4 seconds belong to the BC branch. The possible errors introduced by an occasional use of a PKIKP reading is readily offset by the volume and accuracy of the overall body of data. We show in Fig. 4 histograms of reported arrivals in one degree intervals centred on 145.5°, 150.5° and

153.5°. It is not expected that the DF amplitudes should change significantly between 145° and 153°; thus the dramatic difference in the total count must be attributed to the dominating influence of BC arrivals. At 153.5° all three branches are well separated in time.

The distribution of the points of the CMB crossing is shown in Fig. 5*a* and the resulting model of CMB topography in Fig. 5*b*. Even though the coverage is more even, the expansion is limited to degree 4, to make the direct comparison with the results obtained using the PcP more meaningful. The variance reduction of the PKP_{BC} data set is 10.5% of the original 1.82 s², corresponding to 0.19 s², or 38% of σ_H^2 (see Fig. 2*c*).

Discussion

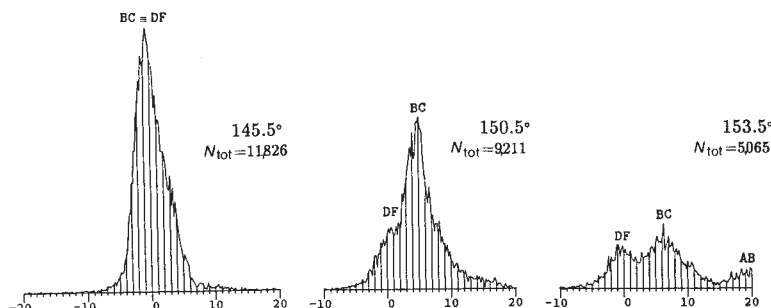
Even visual comparison of Figs 3*b* and 5*b* reveals significant similarity of the two maps in distribution and the sign of the perturbations in the shape of the CMB; the size is somewhat larger in Fig. 5*b*. The correlation coefficient is 0.701 on 23 degrees of freedom; the null hypothesis must be rejected at the confidence level above 99.99%. With the coefficients for degree 1 removed, which likely suffer from errors in earthquake location, the correlation coefficient increases to 0.744 on 20 degrees of freedom. Similarity between the two maps is greater in the Northern Hemisphere, where data coverage is better.

It was noted earlier that the kernels for PcP and PKP are of opposite sign. This means that the compatibility of the two results cannot be explained by a common source of systematic errors, such as the mislocation of sources in space and time or the effect of inaccuracies in our knowledge of the laterally heterogeneous mantle. These effects would result in errors of the same sign for PcP and PKP and, translated in terms of the CMB topography, should lead to maps with reversed polarity. This is not the case and we are led to the conclusion that the shape and magnitude of the anomalies are real.

Another important conclusion is that the level of large-scale lateral heterogeneity in the outer core is below the detectable level, as suggested in earlier studies involving phases PmKP, representing multiple internal reflections in the core²⁶. It seems that, within acceptable errors, the effect inferred from PKP data is also seen in the map derived from PcP observations, and this phase does not enter the core. The PKP_{BC} reaches the bottom of the outer core, and if there was large-scale heterogeneity anywhere in this region, such as proposed by Ritzwoller *et al.*⁵, it would be mapped as a perturbation in the shape of the CMB. Scientists studying the dynamics of the liquid core strongly resist²⁷ suggestions of lateral heterogeneities associated with density differences $|\delta\rho/\rho| > 10^{-5}$. It now seems that this view is supported by seismological evidence.

Creager and Jordan⁶ studied travel-time anomalies of PKIKP and PKP_{AB} using BISC as the data source and projected the residuals, corrected for the mantle structure¹³, onto a region in the vicinity of the CMB. They considered three hypotheses with regard to the source of anomalies: (1) D'' region above the CMB; they reject this hypothesis and our conclusion is the same; (2) perturbations in the radius of CMB and (3) a thin, highly heterogeneous layer under the CMB. They prefer the latter

Fig. 4 Histograms of PKP arrival times in 1° cells as reported in BISC¹⁴. The histograms demonstrate that the preponderance of the arrival times reported in BISC in the distance range from 144° to 154° belong to the BC branch.



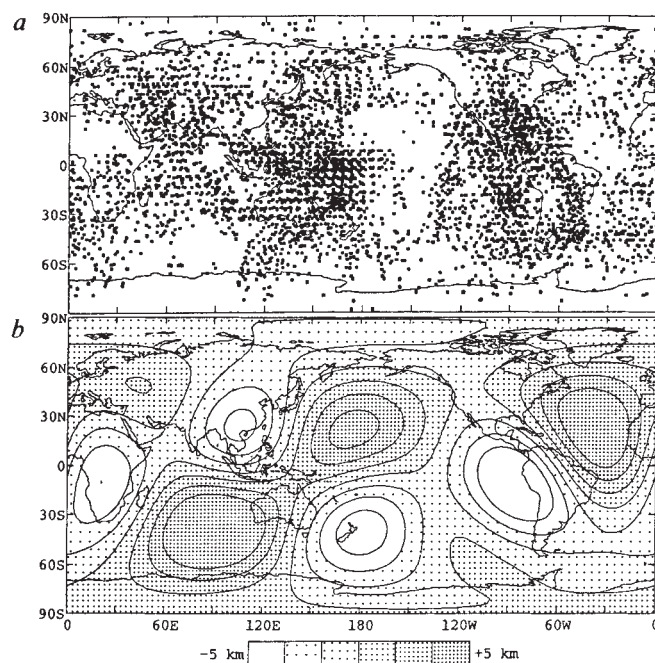


Fig. 5 *a*, Location of points of crossing of the CMB by rays corresponding to the 'summary data' PKP_{BC} used in this study. *b*, Topography of CMB obtained by inversion of PKP_{BC} residuals corrected for lower mantle heterogeneity.

hypothesis, even though their data cannot distinguish between (2) and (3). Their reason for rejection of (2) was the large range of CMB undulations, yet for the PKP_{AB} subset the range of the topography is similar to that proposed here and reported by Gudmundsson *et al.*⁷. The agreement between the results obtained in our report using PcP and PKP_{BC} indicates that (3) should be rejected as the only cause of the travel-time anomalies entering the core. However, the existence of a thin, homogeneous layer below the CMB with properties different (higher viscosity) from that of the bulk of the outer core should be given further consideration. There is an indication from observations of the offset between the figure axis of the earth and the rotational axis of the mantle that the 'astronomical' fluid-solid boundary is smoother than the 'seismic' CMB²⁸. If such a layer was also characterized by a lower P-velocity, this could explain the difference between the amplitude of topography in Figs 3b and

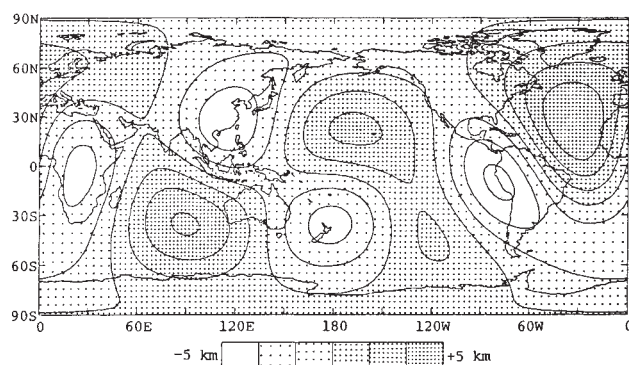


Fig. 6 Topography of CMB obtained by inversion of the combined PcP and PKP_{BC} data set. Spherical harmonic expansion is limited to degree and order 4.

5b. An increase in kernel (2) due to an increase in η_- would lead to a smaller topography in Fig. 5b.

While in some regions the pattern of anomalies of Creager and Jordan is similar to ours, there are also discrepancies. These could have resulted from differences in procedures applied in early stages of data reduction (earthquakes were not relocated in their study, for example).

In view of the compatibility of our results obtained separately from PcP and PKP data, these two sets can be merged and inverted again. The resulting map is shown in Fig. 6, and the coefficients of the spherical harmonic expansion are given in Table 1. They represent our best current estimate of the shape of the CMB. In the future, it may undergo some modification when PKIKP data are included in inversion, but a model of a laterally heterogeneous inner core must be obtained first. The spherically symmetric term implies a correction of -2.5 km with respect to PREM¹²; separate inversions of the PcP and PKP subsets of data yielded similar values. This leads to an estimate of the core radius of $3,477.4 \pm 0.2$ km for the spherically symmetric model, a value very close to $3,477$ km obtained by Taggart and Engdahl²⁹.

The joint inversion does not degrade significantly the fit to the two data sets previously considered separately: the percentage variance reduction for PcP changes from 3.5%, achieved with the model of Fig. 3, to 3.1% with the model of Fig. 6. For PKP_{BC} the value changes from 10.5% to 9.9%.

Hager *et al.*²³ calculated the perturbation in the CMB due to the dynamic load of sinking or rising density anomalies in the mantle; these density anomalies were inferred from aspherical velocity models by assuming the proportionality between density and velocity anomalies. The map in Fig. 6 does not compare favourably with the predictions of Hager *et al.* The flattening of the CMB departs insignificantly from that predicted from the assumption of hydrostatic equilibrium. This is in good agreement with Gwinn *et al.*³⁰ who inferred a departure of 490 ± 110 m from very long baseline interferometry data. The correlation coefficient between the CMB topography and geoid for degrees 2 to 4 is -0.06 . The differences between the results presented here and those of Hager *et al.* suggest that other static or dynamic effects must also be affecting the actual shape of the CMB.

Determination of the shape of the CMB may affect considerations related to the magnetic field of the Earth. Hide and Malin³¹ proposed that 'bumps' at the CMB of size even less than reported here could significantly affect the pattern of flow in the fluid core. Even though his hypothesis has been questioned^{32,33}, it should be realized that we have retrieved only the gravest terms in the expansion. The observed scattering of 10 km wavelength waves at the CMB³⁴ indicates that irregularities may exist over a very wide range of lengths.

The derivation of the perturbations in the shape of the CMB should aid the analysis of PKIKP data in terms of the lateral

Table 1 Spherical harmonic coefficients of the topography of the core-mantle boundary (CMB)

l	m	c_l^m	σ	s_l^m	σ
0	0	-2.50	0.20		
1	0	0.34	0.25		
	1	0.07	0.20	-0.03	0.22
2	0	0.14	0.24		
	1	0.55	0.19	-1.02	0.23
	2	0.29	0.20	0.00	0.20
3	0	-0.26	0.21		
	1	0.30	0.21	-0.04	0.21
	2	1.15	0.20	-0.31	0.17
	3	-0.11	0.20	-1.25	0.19
4	0	-0.11	0.22		
	1	0.22	0.21	0.36	0.20
	2	-0.44	0.22	0.12	0.19
	3	-0.66	0.21	-0.04	0.20
	4	-0.23	0.20	-0.68	0.20

To obtain relief in km, use equation (3); associated Legendre polynomials p_l^m are normalized according to equation (19) of Dziewonski¹³.

heterogeneity of the inner core. The presence of aspherical perturbations in this region of the Earth has been reported in studies using travel times^{3,4,8,35} as well as splitting of normal modes^{1,9}.

Finally, we would like to comment on the distribution of reported PcP arrivals shown in Fig. 3a. The difference in the density of reports cannot be explained by distribution of earthquakes and stations alone. It appears that, with some outstanding exceptions, seismic recordings are not read as diligently south of the Equator as to its north. Global studies, such as this one, are best conducted with a uniform distribution of data. We would like to appeal to the station operators and organizations such as the International Association of Seismology and Physics of the Earth Interior and the International Seismological Centre to encourage efforts that would lead to more frequent reporting of phases sampling in a relatively simple

way the deep interior of the Earth. While it may take hundreds of millions of dollars to map the surface of Venus, the mapping of the more inaccessible interior of our planet could progress much more rapidly at negligible cost. Although there are good prospects that the Earth will be instrumented in the next few years by some 100 high quality broad-band digital stations³⁶⁻³⁸, mostly designed for studies of waveforms, they will not replace the contribution that can be made by a careful analysis of seismograms at several thousand existing installations.

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- Giardini, D., Li, X.-D. & Woodhouse, J. H. *Nature* **325**, 405-411 (1987).
- Woodhouse, J. H. & Dziewonski, A. M. *Trans. Am. geophys. Un.* **67**, 307 (1986).
- Morelli, A. & Dziewonski, A. M. *Trans. Am. geophys. Un.* **67**, 311 (1986).
- Poupinet, G., Pillet, R. & Souriau, A. *Nature* **305**, 204-206 (1983).
- Ritzwoller, M., Masters, G. & Gilbert, F. *J. geophys. Res.* **91**, 10203-10228 (1986).
- Creager, K. C. & Jordan, T. H. *Geophys. Res. Lett.* **13**, 1497-1500 (1986).
- Gudmundsson, O., Clayton, R. W. & Anderson, D. L. *Trans. Am. geophys. Un.* **67**, 1100 (1986).
- Morelli, A., Dziewonski, A. M. & Woodhouse, J. H. *Geophys. Res. Lett.* **13**, 1545-1548 (1986).
- Woodhouse, J. H., Giardini, D. & Li, X.-D. *Geophys. Res. Lett.* **13**, 1549-1552 (1986).
- Dziewonski, A. M. & Gilbert, F. *Geophys. J. R. astr. Soc.* **44**, 7-16 (1976).
- Jeffreys, H. *Mon. Nat. R. astr. Soc., Geophys. Suppl.* **4**, 537-547 (1939).
- Dziewonski, A. M. & Anderson, D. L. *Phys. Earth planet. Interior* **25**, 297-356 (1981).
- Dziewonski, A. M. *J. geophys. Res.* **89**, 5929-5952 (1984).
- Bulletin of the International Seismological Centre: Catalogue of Events and Associated Observations (Years 1964-1982)*, Vols. 1-19 (International Seismological Centre, Newbury, UK, 1967-1984).
- Dziewonski, A. M. *Trans. Am. geophys. Un.* **56**, 395 (1975).
- Dziewonski, A. M., Hager, B. H. & O'Connell, R. J. *J. geophys. Res.* **82**, 239-255 (1977).
- Clayton, R. W. & Comer, R. P. *Trans. Am. geophys. Un.* **64**, 776 (1983).
- Zhou, H.-W. & Clayton, R. W. *Trans. Am. geophys. Un.* **66**, 975 (1985).
- Toy, K. M., Olson, A. H. & Orcutt, J. A. *Trans. Am. geophys. Un.* **67**, 1099 (1986).
- Davies, J. H. & Clayton, R. W. *Trans. Am. geophys. Un.* **67**, 1099 (1986).
- Dziewonski, A. M. & Anderson, D. L. *J. geophys. Res.* **88**, 3295-3314 (1983).
- Morelli, A. & Dziewonski, A. M. *Trans. Am. geophys. Un.* **66**, 975 (1985).
- Hager, B. H., Clayton, R. W., Richards, M. A., Comer, R. P. & Dziewonski, A. M. *Nature* **313**, 541-545 (1985).
- Richards, P. G. *Geophys. J. R. astr. Soc.* **35**, 243-264 (1973).
- Andersen, R. S. & Cleary, J. R. *Phys. Earth planet. Inter.* **23**, 207-214 (1980).
- Buchbinder, G. G. *R. Earth planet. Sci. Lett.* **14**, 161-168 (1972).
- Stevenson, D. *Geophys. J. R. astr. Soc.* (in the press).
- Wahr, J. M. *Geophys. J. R. astr. Soc.* (submitted).
- Taggart, J. N. & Engdahl, E. R. *Bull. seism. Soc. Am.* **58**, 1293-1303 (1988).
- Gwinn, C. R., Herrin, T. A. & Shapiro, I. I. *J. geophys. Res.* **91**, 4755-4765 (1986).
- Hide, R. & Malin, S. R. C. *Nature* **255**, 606-609 (1970).
- Eckhardt, D. H. *Math. Geol.* **16**, 155-171 (1984).
- Anufriyev, A. P. & Braginsky, S. I. *Geomagn. Aeron.* **17**, 492-496 (1977).
- Cleary, J. R. & Haddon, R. A. W. *Nature* **240**, 549-551 (1972).
- Cornier, V. F. & Choy, G. L. *Geophys. Res. Lett.* **13**, 1553-1556 (1986).
- Romanowicz, B., Cara, M., Fels, J. F. & Rowland, D. *Trans. Am. geophys. Un.* **65**, 753 (1984).
- Smith, S. W. *Trans. Am. geophys. Un.* **67**, 213-219 (1986).
- Romanowicz, B. & Dziewonski, A. M. *Trans. Am. geophys. Un.* **67**, 541-542 (1986).

A T-cell receptor γ /CD3 complex found on cloned functional lymphocytes

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Cloned blood lymphocytes that do not express the α - and β -chains of the T-cell receptor show MHC-unrestricted cytotoxicity. These cells carry the γ -protein, disulphide-linked either to another molecule or to itself, and associated with the CD3 complex. These observations may help to solve the mystery posed by the discovery of the γ gene.

T LYMPHOCYTES characteristically recognize foreign antigens in the context of molecules of the major histocompatibility complex (MHC). The molecular basis of this recognition has become amenable to investigation by virtue of the identification of the T-cell receptor (TCR) molecule, both at the protein¹⁻³ and the DNA⁴⁻⁶ level. This molecule consists of two disulphide-linked chains, α and β , each containing a variable and a constant domain⁷⁻⁹, encoded in different gene segments (V , D , J and C)^{4-6,10} that join through rearrangement during T-cell ontogeny. The TCR heterodimer is physically linked at the cell surface with the CD3 (T3) protein complex¹¹⁻¹⁴, which consists of at least three different, non-polymorphic subunits^{12,15}. Association with the CD3 complex is a prerequisite for surface expression of the TCR α and β chains^{16,17}. The TCR $\alpha\beta$ molecule acts as

the recognition unit determining T-cell specificity for antigen plus MHC molecules and the CD3 molecule may be important in subsequent signal transduction through the cell membrane, as reviewed in ref. 18.

Along with the TCR α and β genes, the putative TCR γ gene was identified; it rearranges specifically in T cells which are either cytotoxic^{6,19} or helper cells²⁰. The γ gene is organized in the same way as the α and β TCR genes²¹, but its diversity may be more limited²². In man, at least ten variable (V), five joining (J), and two constant (C) gene segments have now been found (refs 22-28 and J.S. *et al.*, unpublished results). The TCR γ gene is rearranged and expressed relatively early in T-cell ontogeny, preceding α -gene rearrangement²⁹⁻³¹. Recently, the protein product of the TCR γ gene has been identified³² on peripheral blood lymphocytes (PBL) derived from immunodeficiency patients. The γ protein may also be expressed on CD4⁺8⁻

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Table 1 Characteristics of lymphocyte clones

Clone	Origin	WT31	CD3	Cell surface marker		CD2	CD16	ADCC
				CD4	CD8			
AK 4	Normal* PB†	—	+	—	—	+	+	+
AK 615	Normal PB	—	+	—	—	+	+	+
AK 925	SCID PB	—	+	—	—	+	+	+
AK 119	Cancer PE†	—	+	—	—	+	—	—
AK 781	Normal* PB	+	+	—	—	+	—	—
CTL 20	Normal* PB	+	+	—	+	+	—	—
CAK 11	Normal PB	+	+	—	+	+	—	—
NK 77	T γ -lymphocytosis PB	—	—	—	—	+	+	+

Cells were cultured by weekly stimulation with a feeder cell mixture consisting of irradiated EBV-transformed B cells and PBL as described previously³⁷. Monoclonal antibodies used were OKT3 (anti-CD3), OKT4 (anti-CD4), OKT8 (anti-CD8), OKT11 (anti-CD2) all obtained from Ortho, VD2 (anti-CD16) obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and WT31 (anti-TCR framework³⁸), obtained from Dr W. Tax. Fluorescence was determined using a fluorescence-activated cell sorter (FACS II, Becton-Dickinson). Expression of the IgG-FcR as measured with the VD2 monoclonal antibody was always lower on CD3⁺WT31[—] clones than on CD3[—] NK cells.

* Same individual; † PB, peripheral blood; PE, pleural effusion.

(T4[—]T8[—]) thymocytes³³. Strikingly, the TCR γ -gene product occurs in association with the CD3 complex at the surface of T cells that do not express the α , β receptor^{32,33}. So far, the role of the TCR γ protein in T-cell function has remained unclear.

We have previously described a small population (~2%) of peripheral CD3⁺ T cells that are CD4[—]8[—]^{34,35}, a phenotype also found in the thymus^{33,36}. We have now generated CD3⁺4[—]8[—] T cell clones from peripheral blood and describe here the phenotypic and functional characteristics of these cells; they express CD3 molecules, but most of them lack clone-specific TCR α and β molecules. Moreover, unlike most mature CD3⁺4⁺ or CD8⁺ MHC-restricted cytotoxic T cells (CTL), but like CD3[—] natural killer (NK) cells, CD3⁺4[—]8[—] cloned lymphocytes have MHC non-restricted cytolytic capacity and can mediate antibody dependent cellular cytotoxicity (ADCC). The CD3 molecule is involved in the regulation of cytotoxicity. These clones lack productive α and β TCR gene rearrangements, but they do rearrange and express TCR γ genes. At the cell surface these CD3⁺4[—]8[—] T-cell clones express CD3-associated disulphide-linked heterodimers, comprising the γ protein and a non- γ protein that does not react with heterologous anti- γ serum. Our findings raise the possibility that this novel TCR is involved in MHC non-restricted cytotoxicity by T cells that use CD3 for signal transduction.

Cytotoxic function

Lymphocytes were cloned by limiting dilution³⁷ from PBL of healthy donors, of a patient with severe combined immunodeficiency (SCID), and from pleural effusion fluid of a patient with ovarian cancer. Five CD3⁺4[—]8[—] clones were studied in detail (Table 1). Strikingly, four out of five clones lacked the common epitope of the TCR heterodimer as defined by the monoclonal antibody WT31 (ref. 38), but did express the CD3 antigen. Indeed, 65% of CD4[—]8[—] FACS-selected PBL were found to be CD3⁺, and only 10% of those reacted with WT31 antibody.

CD3⁺4[—]8[—] cells freshly isolated from peripheral blood do not show substantial lytic activity towards K562 target cells³⁹, but after cloning and expansion in feeder cell mixture³⁷ they acquired cytolytic activity against a variety of tumour cells. The target cell spectra of CD3⁺4[—]8[—]WT31[—] clones AK 615, 4 and 119 are given and compared with those of: (1) a CD3⁺4[—]8[—]WT31⁺ clone AK781; (2) a CD3⁺4[—]8[—]WT31⁺ CTL clone CAK 11, which interacts with class I MHC; (3) a CD3⁺4[—]8[—]WT31[—] NK cell-derived clone NK 77 (Fig. 1). At effector to target cell (E:T) ratios of 3:1 and 27:1 the CD3⁺WT31⁺ clones AK 781 and CAK 11 showed only limited MHC non-restricted cytotoxicity, as observed earlier⁴⁰. In contrast, the NK cell-derived clone NK 77 efficiently lysed K562, Daudi, monocytic and T-cell lines and a small cell lung carcinoma line (GLC1) at both E:T ratios. The CD3⁺4[—]8[—] WT31[—] clones

AK 615, 4 and 119 lysed a variety of tumour cells at the E:T ratio 27:1, but Daudi cells were not lysed by AK 119. At the E:T ratio 3:1, the CD3⁺WT31[—] clones showed a more selective and individual target cell spectrum.

As summarized in Table 1 and detailed elsewhere⁴¹ three out of four CD3⁺4[—]8[—]WT31[—] cloned cells also showed ADCC, a characteristic of NK cells expressing receptors for the Fc portion of immunoglobulin G (IgG-FcR; CD16). This finding functionally confirms the reactivity of the CD3⁺WT31[—] clones with monoclonal antibodies directed against the IgG-FcR (Table 1).

CD3 regulates cytolytic activity

The anti-CD3 monoclonal antibody WT32 inhibited the lytic activity of CD3⁺WT31[—] clones AK 4 and 615 against target cells Daudi and GLC1, but induced cytotoxicity of clones AK 4, 615 and 119 provided target cells were used that express FcR for the IgG_{2a} monoclonal antibody WT32 (U937 and P815; Table 2). This antibody did not induce cytotoxicity by clone AK 119 of Daudi, which does not express FcR for IgG_{2a}. Induction of lysis has previously been described for MHC class I- or II-specific T-cell clones⁴²⁻⁴⁴.

Thus, we have demonstrated a unique category of CD3⁺4[—]8[—]WT31[—] peripheral T cells. These cells share features with mature T cells in that they express the CD3 antigen, which is involved in the regulation of their lytic activity; however, they lack surface expression of the TCR $\alpha\beta$ heterodimer. On the other hand, the clones also share features with CD3[—] NK cells, in that they display MHC non-restricted lysis, bear IgG-FcR and can mediate ADCC. Moreover, the clones also respond to the addition of recombinant interleukin-2 and β -interferon with augmented lytic activity⁴¹.

Table 2 Anti-CD3 monoclonal antibody modulates the cytotoxicity of CD3⁺4[—]8[—]WT31[—] clones

Clone	WT32*	% Specific ⁵¹ Cr release from target cell			
		Daudi	GLC1	U937	P815
AK 4	—	44†	15	25	0
	+	16	4	45	62
AK 615	—	55	24	—	—
	+	13	10	—	—
AK 119	—	0	—	0	0
	+	0	—	55	31

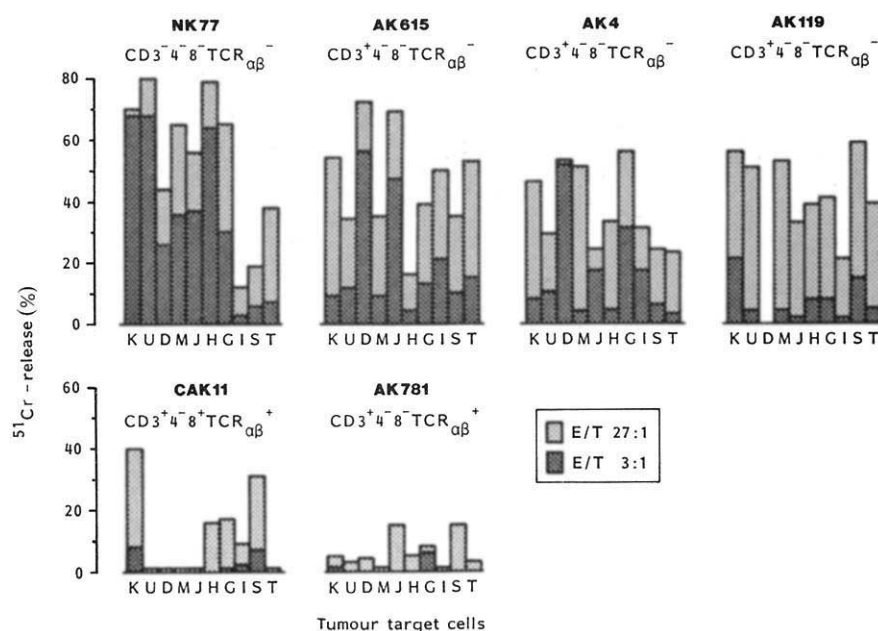
Target cells Daudi, GLC1 and U937 are described in Fig. 1. P815 is a murine mastocytoma cell line.

* WT32 antibody (IgG_{2a}) detects the CD3 antigen⁵⁵ and was obtained from Dr W. Tax. Effector cells were incubated for 30 min with 1:100 dilution of WT32 ascites fluid before assay.

† Percentage ⁵¹Cr-release in a 3-h cytotoxicity assay. Effector to target cell ratio was 3:1.

Fig. 1 MHC non-restricted cytolytic activity of $CD3^+WT31^-$, $CD3^+WT31^+$ and $CD3^-WT31^-$ clones. Cytolytic activity was determined in a 3-h ^{51}Cr -release assay in round-bottom microtitre plates. Characteristics of clones are outlined in Table 1. Tumour target cell lines: K, K562 (erythromyeloid); U, U937 (monocytic cell line); D, Daudi (B cell line); M, MOLT-4, J, Jurkat and H, HSB (T cell lines); G, GLC1 (from small cell lung carcinoma); I, IGR37 and S, SKMEL (melanoma derived); T, T24 (from bladder carcinoma).

E:T, effector: target cell ratio.



TCR γ messenger RNA is expressed

We next investigated whether the TCR $\alpha\beta$ heterodimer was expressed on the $CD3^+WT31^-$ T-cell clones in a form not detectable by antibody WT31. RNA was isolated from the $CD3^+WT31^-$ clones AK 4, 615 and 119, and, as positive controls, from the $CD3^+WT31^+$ clone AK 781 and the T leukaemic cell lines Jurkat and HPB-ALL (Fig. 2). Mature α mRNA was present in Jurkat, HPB-ALL and clone AK 781, but was not found in clones AK 4, 119 and 615. No mature 1.3-kilobase (kb) β chain mRNA was found in clones AK 4, 119 and 615, but this message was expressed in Jurkat, HPB-ALL and clone AK 781. In clone AK 4 and (upon longer exposure of the autoradiogram) also in clones AK 119 and 615, a 1.0-kb β -chain message was detected, representing a defective transcript. We conclude that these $CD3^+WT31^-$ clones do not express TCR α and β chains.

As these cells lack TCR $\alpha\beta$ expression but have a functional CD3 molecule they were candidates for the expression of the TCR γ protein. Clones AK 4, 119 and 615 do synthesize TCR γ mRNA, which is not produced in the $CD3^+WT31^+$ clone AK 781 (Fig. 2). No rearrangement of the α chain gene was found in the $CD3^+WT31^-$ clones, although it cannot be excluded that a non-functional rearrangement had taken place but had remained undetected (unpublished results). A rearranged β -chain gene could be demonstrated in $CD3^+WT31^-$ clone AK 4, but not in clones AK 925, 615 and 119 (Fig. 3a and data not shown). This rearrangement in clone AK 4 probably gives rise to the 1.0-kb mRNA observed in Fig. 2. As expected, rearrangements of both α and β chain genes were detected in the $CD3^+4^-8^-WT31^+$ clones AK 781 and CAK 11, but not in a $CD3^-$ NK-cell derived clone NK 77, consistent with previous reports^{45,46}.

Rearrangement of the TCR γ gene was studied by hybridization of a *Bam*HI digest with a genomic probe specific for the $J_{\gamma 1.3}$ gene segment (nomenclature and probe according to ref. 47). Only one γ -gene rearrangement was observed in DNA from $CD3^+WT31^-$ clones AK 615, 119 and 925 (Fig. 3b). The *Bam*HI digest of DNA from $CD3^+WT31^-$ clone AK 4 did not demonstrate an easily identifiable γ -gene rearrangement (not shown), but the $CD3^+WT31^+$ clones CTL 20 and AK 781 had undergone γ -gene rearrangements on both chromosomes. Thus, the $CD3^+4^-8^-WT31^-$ clones may have undergone significantly less gene rearrangement than the $CD3^+WT31^+$ clones shown here, as well as leukaemic cells of such phenotype examined recently^{23,26,48,49}.

In conclusion, $CD3^+WT31^-$ clones: (1) may have unrearranged

TCR α genes and do not express α mRNA; (2) may have rearranged TCR β genes but only to the extent that a non-functional, truncated mRNA can be made; (3) undergo γ gene rearrangement and produce mature γ mRNA.

CD3-associated disulphide-linked dimers

To determine whether the CD3 molecule present on the $CD3^+WT31^-$ clones was associated with molecules other than the TCR $\alpha\beta$ heterodimer, the $CD3^+WT31^-$ clones AK 4, 615, 119 and 925 and the $CD3^+WT31^+$ clones CTL20 and AK 781 were surface-labelled with $Na^{125}I$. Immunoprecipitates were made using monoclonal anti-CD3 antibody and analysed by SDS-PAGE. Under both non-reducing and reducing conditions, the CD3 glycoproteins of relative molecular mass 19,000–21,000

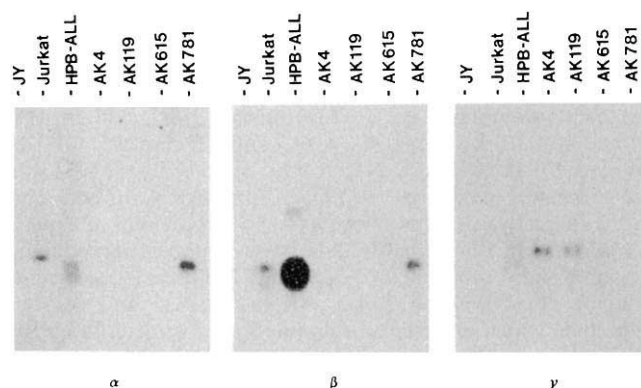


Fig. 2 Expression of TCR $\alpha\beta$ and γ genes in $CD3^+WT31^-$ clones. Total cellular RNA was extracted according to the LiCl-urea method and electrophoresed at 10–20 μ g per lane on a formaldehyde-containing 1.3% agarose gel. RNA was transferred to nitrocellulose and the same blot was probed three times with ^{32}P -labelled β , α and γ chain probes in the form of isolated inserts, in that order. Probes were removed by washing the blot for 5 min in 0.1 M NaOH, followed by neutralization in 10 mM Tris-HCl, pH 7.5, $3\times$ SSC. Removal of the previously used probe was checked by autoradiography. The α -chain probe used was isolated from a complementary DNA library of HPB-MLT and kindly provided by Dr J. L. Strominger. The β -chain probe was HPB- β 2, isolated from a cDNA library of HPB-ALL and a gift from Dr C. P. Terhorst. The γ chain probe was pTy-1, isolated from an HPB-MLT cDNA library as described²⁸. This method allows qualitative, but not quantitative measurement of mRNA expression.

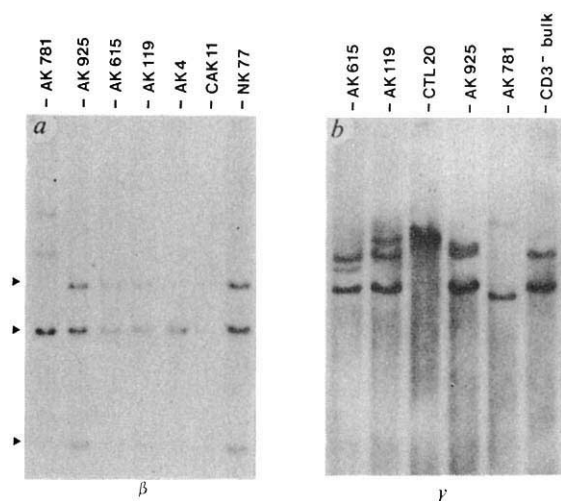


Fig. 3 Rearrangement of *a*, TCR β and *b*, TCR γ genes in CD3⁺WT31⁺ clones. High M_r DNA was extracted according to established procedures, digested to completion with the restriction endonucleases *Hind*III (β) or *Bam*HI (γ), electrophoresed on 0.6% agarose gels, and transferred to nitrocellulose sheets. Hybridization was performed at 42°C in buffer containing formamide and 10% w/v dextran sulphate. Filters were washed at 55°C, reducing the salt concentration of the wash buffer by step from $3 \times$ SSC to $0.1 \times$ SSC, at 0.1% SDS. The human TCR β chain probe used was an *Eco*RI-*Hind*III 2-kb genomic DNA fragment containing the $C_{\beta 2}$ constant region gene segment. This probe was a gift from Dr T. H. Rabbitts. The TCR γ probe was a genomic *Eco*RI-*Hind*III 0.8 kb fragment containing $J_{\gamma 1.3}$ (ref. 27). Arrows indicate germline configuration of β and γ genes.

(M_r 19–21K) (CD3 δ) and 22–25K (CD3 γ) can be seen, resolving as diffuse bands because of variations in the extent of glycosylation¹² (Fig. 4a–d). Under non-reducing conditions (Fig. 4a, b) an additional band was found of M_r 80K in the CD3⁺WT31⁺ clones CTL 20 and AK 781, of 70K in CD3⁺WT31⁺ clone AK 119 and 75K in CD3⁺WT31⁺ clones AK 4, 615 and 925. In CD3⁺WT31⁺ clones this 80K band is the disulphide-linked TCR α and β dimer^{1–3}. Under reducing conditions (Fig. 4c, d), in CTL 20 and AK 781 the TCR α and β subunits are resolved independently, with M_r 46K and 36K and 44K and 39K respectively. In the CD3⁺WT31⁺ clones two prominent bands were found under reducing conditions with M_r 43K (1) and 40K (2) in clones AK 4, 615 and 925 and M_r 40K (2) and 36K (3) in clone AK 119. A third band with M_r 36K was also present in samples derived from clones AK 4, 615 and 925, but its intensity varied between experiments (compare experiments I and II). Given the M_r of the bands found under reducing conditions, the molecules of 75K and 70K seen under non-reducing conditions on clones AK 4, 615, 925 and AK 119 respectively must represent disulphide-linked dimers. In clones AK 4, 615 and 925 only the 43K and the 40K molecules may be required for dimer formation, as the dimer is also present when the 36K band is not detectable (see experiment I). In clone AK 119 the dimer could be composed of the 40K and 36K subunits.

The dimers contain the TCR γ chain

A rabbit antiserum raised against amino acids 137–157 of the TCR γ sequence^{28,32} was used to determine whether the TCR γ gene product participated in the dimeric CD3-associated molecule on the CD3⁺WT31⁺ clones. Cell lysates were mildly denatured before immunoprecipitation, which allows detection of the γ protein, but also disrupts the interactions between CD3 and the γ components³². Figure 4d shows that the anti-C γ serum precipitated two bands with M_r 40K and 36K from the four CD3⁺WT31⁺ clones. In contrast, no specific bands were isolated from the CD3⁺WT31⁺ $\alpha\beta$ ⁺ clone AK 781. This demonstrates

that the protein product of the TCR γ gene is synthesized in the CD3⁺WT31⁺ clones and strongly suggests that the 40K and 36K subunits associated with CD3 (Fig. 4a–d) represent the TCR γ proteins.

To prove this point, anti-CD3 immunoprecipitates were made from clones AK 781, 4 and 119 and treated so as to dissociate the protein complex from the antibody and partly denature its components³². Immunoprecipitation was then performed using anti-C γ serum. Figure 4e shows that the 40K and 36K components previously associated with CD3 were now selectively isolated, whereas no material was precipitated from an anti-CD3 sample derived from CD3⁺WT31⁺ clone AK 781. This shows that the 40K and 36K CD3-associated subunits bear TCR γ determinants. But the anti-C γ serum did not identify a 43K protein in the CD3⁺WT31⁺ clones, although a molecule of this M_r was found to be associated with CD3 in clones AK 4, 615 and 925. Thus in clones AK 4, 615 and 925 the dimers are composed of a γ chain and a 43K non- γ chain. In clone AK 119, the dimer might be composed of two γ chains of 40K and 36K or, alternatively, of a γ chain and another component similar to the 43K subunit found in the other clones, which remained undetected for unknown reasons.

Relationship between 40K and 36K TCR γ chains

The 40K and 36K γ chains were further investigated by two-dimensional gel electrophoresis (isoelectric focusing followed by SDS-PAGE). Anti-CD3 precipitates made from CD3⁺WT31⁺ clone AK 119 and from CD3⁺WT31⁺ clone AK 781 as a control, were analysed before and after treatment with endoglycosidase F (endo F), which cleaves both high mannose and complex N-linked carbohydrates⁵⁰. Before endo F treatment, TCR α and β chains isolated from clone AK 781 (M_r 44K and 39K) as well as CD3 components (M_r 19–25K) were heterogeneous in charge, due to the presence of sialic acids¹². After deglycosylation the chains were virtually homogeneous in charge, the α chain being acidic and the β chain more basic. The CD3-associated 40 and 36K bands from clone AK 119 had very similar isoelectric focusing patterns, showing the close relationship between the two bands. After endo F treatment there was no difference in M_r between the two γ components. But a spot remained with an isoelectric point (pI) of ~ 8 that was also found before endo F treatment, and a slightly less basic spot that was probably the result of desialation of the molecules found in the two heterogeneous smears before endo F treatment.

Therefore, differential glycosylation causes the molecular weight difference found for the two γ chains in clone AK 119. However, the relationship between the two spots remaining after endo F treatment is not clear.

Discussion

Brenner *et al.* have recently reported CD3⁺4⁺8⁺ or 4⁺8⁺ cells derived from PBL of immunodeficiency patients that lack the $\alpha\beta$ TCR and express a CD3-associated TCR γ protein³². Bank *et al.* described a cloned cell line derived from CD3⁺4⁺8⁺ thymocytes, which may express a CD3-associated TCR γ chain³³. These cells showed no lytic activity unless treated with anti-CD3 monoclonal antibody.

We reported that a small population ($\sim 2\%$) of PBL obtained from normal donors shows the CD3⁺4⁺8⁺ phenotype^{34,35}. Most of these cells do not react with the anti- $\alpha\beta$ TCR monoclonal antibody WT31. Clones of this CD3⁺4⁺8⁺ WT31⁺ phenotype derived from PBL of normal donors, of a SCID patient and from pleural effusion of a cancer patient were found to be functionally active: they can exert MHC non-restricted cytotoxicity and ADCC. These clones also express CD3-associated TCR γ chains. Clones of CD3⁺WT31⁺ phenotype have also been isolated from fetal PBL, but the nature of their receptor is as yet unknown⁵¹.

The question is how these TCR γ -expressing CD3⁺4⁺8⁺ cells fit into T cell differentiation pathways. Fetal thymocytes which

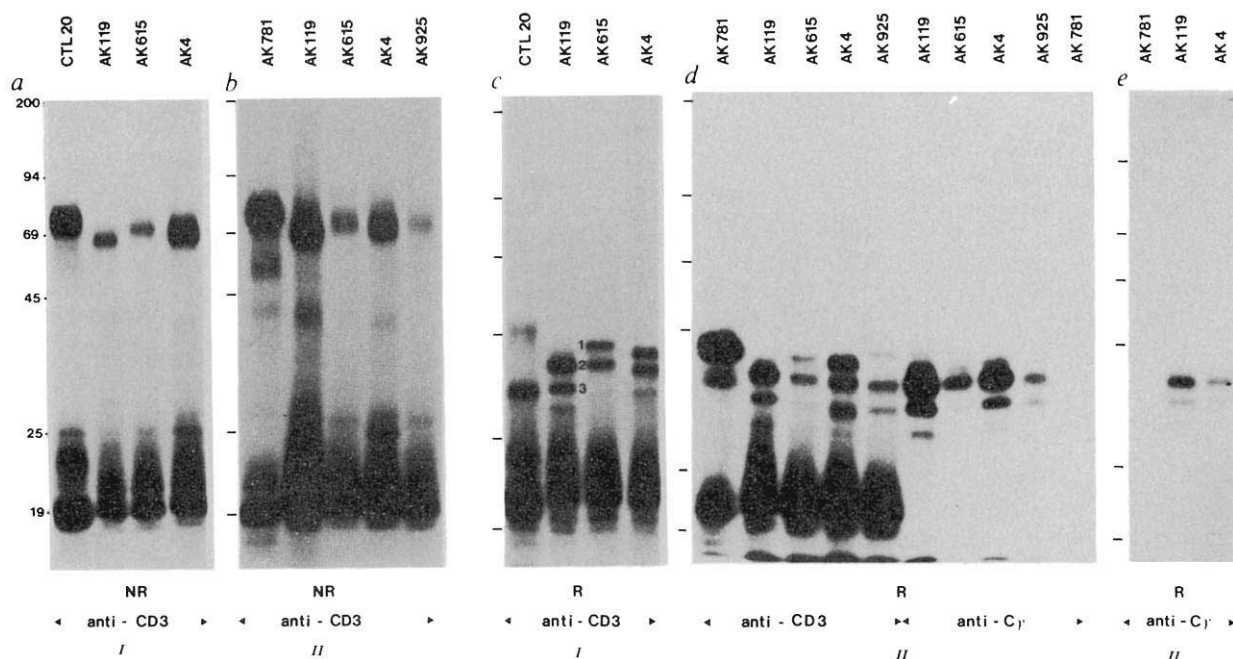


Fig. 4 SDS-PAGE of anti-CD3 and anti-C γ immunoprecipitates made from ^{125}I -labelled CD3 $^{+}$ WT31 $^{-}$ and CD3 $^{+}$ WT31 $^{+}$ clones. About 20×10^6 cells were surface labelled with Na^{125}I using 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen, Pierce Chemical Co.) as a catalyst. Cells were lysed in 0.01 M triethanolamine-HCl, pH 7.8, 0.15 M NaCl, 1 mM phenylmethylsulphonylfluoride (PMSF), and 0.02 mg ml $^{-1}$ ovomucoid trypsin inhibitor (TI) (immunoprecipitation buffer, IPB), containing 1% digitonin 56 (Sigma) in the case of anti-CD3 immunoprecipitates, or 1% Nonidet P40 (NP40) for anti-C γ immunoprecipitates. Lysates were precleared and anti-CD3 immunoprecipitation (a-d) was done with monoclonal anti-Leu-4 antibody coupled covalently to protein-A CL4B Sepharose beads with dimethylpilmidate-HCl. Beads were washed five times in IPB, 1% digitonin. For anti-C γ immunoprecipitation (d), 1% SDS and 2 mM dithiothreitol (DTT) were added to the lysates, which were incubated for 5 min at 68 $^{\circ}\text{C}$. Subsequently, samples were diluted with five volumes IPB, 1.5% NP40 and iodoacetamide was added to 20 mM. Lysates were precleared, anti-C γ serum was added and immune complexes were recovered with protein-A Sepharose beads. Beads were washed five times in IPB, containing either 0.5% deoxycholate or 0.5% NP40. Samples were analysed on SDS-polyacrylamide gradient gels, either under non-reducing (NR, 1 mM iodoacetamide), or under reducing conditions (R, 5% v/v 2-mercapto-ethanol). I, samples derived from labelling I; II, samples derived from labelling II. For reprecipitation (e), anti-Leu-4 precipitates were incubated in 100 μl IPB containing 1% SDS and 2 mM DTT for 5 min at 68 $^{\circ}\text{C}$. Beads were spun out and the supernatant was diluted to 500 μl with IPB, then 1.5% NP40, 20 mM iodoacetamide and 25 μg myoglobin were added and the supernatant was precleared once with normal mouse immunoglobulin-coated beads, after which anti-C γ immunoprecipitation was performed as outlined above. 1, 43K band; 2, 40K band; 3, 36K band. Size markers are M_r in thousands.

lack TCR α mRNA can express CD3 δ and ϵ subunits 52 . This finding, combined with the fact that cells expressing significant levels of TCR γ message are present at an early stage of T-cell development in the thymus, suggests that certain early thymocytes may express γ /CD3 protein complexes at the cell surface. Indeed, such cells were reported by Bank *et al.* 33 . Therefore we postulate that the CD3 $^{+}$ 4 $^{-}$ 8 $^{-}$ WT31 $^{-}$ cells found in peripheral blood undergo TCR γ gene rearrangement in the thymus, start expressing a γ /CD3 complex at the cell surface, and are subsequently exported as mature T cells.

In the CD3 $^{+}$ WT31 $^{-}$ clones we observed rearrangement in only one allele of the TCR γ gene. Most human leukaemic T cell lines (48 out of 51) have gene rearrangement involving both γ gene alleles 23,26,48,49 . These rearrangements do not lead to γ protein expression in these cells but the TCR α and β chains are expressed. Therefore, we propose a model of T-cell differentiation in which the γ gene rearranges first on one chromosome and next on the other until a functional gene is formed leading to a TCR involving the γ protein. Such cells may represent a distinct mature T cell subset. Cells that fail to express γ chain protein then productively rearrange and express the TCR α and β genes, eventually producing a classic CD3-associated $\alpha\beta$ TCR.

Here we show conclusively that CD3-associated disulphide-linked dimeric molecules that involve the TCR γ protein exist. Brenner *et al.* have reported a CD3-associated γ protein, non-covalently linked to a different, non- γ protein, which may form a putative second TCR 32 . Apparently, the γ protein is more versatile in its quaternary organization than the TCR α and β

chains, which are invariably disulphide-linked. It is tempting to speculate that this difference in molecular organization is related to the differentiation stage and to the functional capabilities of the cells on which the TCR γ protein is expressed. We find clear indications of two different types of dimers: three clones express 40K and 36K chains which bear γ determinants, disulphide-linked to an additional 43K non- γ chain. One clone (AK 119) expresses only the 40 and 36K γ chains, which are probably disulphide-linked to one another, as the dimer on this clone has a slightly lower M_r . However, the possibility exists that the 43K non- γ chain has remained undetected. We suspect that the 40K and 36K chains bearing the γ epitopes are differentially processed protein products of the same gene; only one γ gene is rearranged in these clones. Moreover, one-dimensional peptide mapping according to Cleveland indicates extensive homology between these chains (results not shown). The nature of the 43K non- γ chain needs further study. The M_r of the CD3-associated chains differ from those reported by Brenner *et al.* 32 , who found a γ chain of M_r 55K and a non- γ chain of 40K, but this may result from differences in glycosylation. Indeed, the human γ chain may harbour five, or possibly more (depending on V gene use) sites for N-linked carbohydrates 33 . It is interesting in this respect that Weiss *et al.* 53 have recently identified a CD3-associated chain that might be γ , with M_r 55-60K and a polypeptide backbone of 29K.

This is the first report suggesting a link between expression of the TCR γ protein and cytotoxic activity. At high E:T ratios the CD3 $^{+}$ WT31 $^{-}$ clones display cytotoxic activity towards a wide range of target cells, but at low E:T ratios the lytic patterns

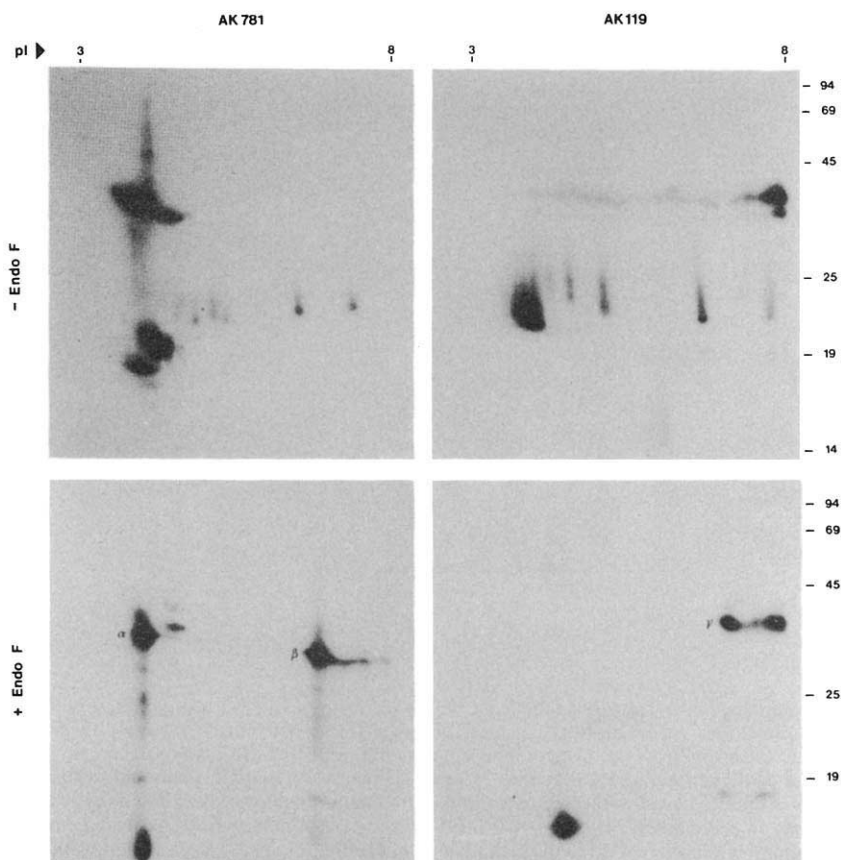


Fig. 5 Two-dimensional gel electrophoresis according to O'Farrell⁵⁷ of glycosylated and deglycosylated anti-CD3 immunoprecipitates derived from clones AK 781 and 119. Samples were immunoprecipitated as described in Fig. 4. Deglycosylation with endo F⁵⁰ was performed as described¹². Two-dimensional gel electrophoresis was performed using ampholytes (LKB, Bromma, Sweden) of pI 3.5–10, 4–6, 5–8 and 9–11 as 10:1:1:1. The second dimension was a 10–15% SDS polyacrylamide gel. Size markers, are M_r in thousands.

become more selective, in contrast to those of NK clones. The γ protein is associated with the CD3 antigen, which is analogous to the organization of the $\alpha\beta$ TCR. Cytolytic activity of both types of clones, bearing either an $\alpha\beta$ or γ TCR, is modulated by anti-CD3 antibodies, in contrast to that of CD3⁺ NK cells. In view of its structural variability it would be anticipated that the γ protein on these cytolytic cells has a specific recognition function. Although a number of different tumour target cells were used here, the results obtained do not allow us to discriminate between the apparent MHC non-restricted cytotoxicity of CD3⁺4⁺8⁺ γ expressing T cells and that of CD3⁺ NK cells. More detailed analysis of the cytolytic patterns of the clones, using target cell panels of distinct histogenetic origins, may

provide insight into their presumed specificities.

Considerable evidence is available to ascribe distinct functional roles to CD3⁺NK cells and CD3⁺TCR $\alpha\beta$ ⁺ T cells in the immune response⁵⁴. The CD3⁺ TCR γ ⁺ T cells described here may constitute a separate T-cell population, with a distinct immune function.

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- Allison, J. P., McIntyre, B. W. & Bloch, D. J. *J. Immun.* **129**, 2293–2300 (1982).
- Haskins, K. *et al. J. exp. Med.* **157**, 1149–1169 (1983).
- Meuer, S. C. *et al. J. exp. Med.* **157**, 705–719 (1983).
- Yanagi, Y. *et al. Nature* **308**, 145–149 (1984).
- Hedrick, S. M., Cohen, D. I., Nielsen, E. A. & Davis, M. M. *Nature* **308**, 149–153 (1984).
- Saito, H. *et al. Nature* **309**, 757–762 (1984).
- Acuto, O. *et al. Cell* **34**, 717–725 (1983).
- Kappler, J. W. *et al. Cell* **35**, 295–302 (1983).
- McIntyre, B. W. & Allison, J. P. *Cell* **34**, 739–746 (1983).
- Kronenberg, M., Siu, G., Hood, L. & Shastri, N. A. *Rev. Immun.* **4**, 529–591 (1986).
- Reinherz, E. L. *et al. Cell* **30**, 735–743 (1982).
- Borst, J., Alexander, S., Elder, J. & Terhorst, C. *J. biol. Chem.* **258**, 5135–5141 (1983).
- Oetting, H. C., Kappler, J., Tax, W. J. M. & Terhorst, C. *J. biol. Chem.* **259**, 12039–12048 (1984).
- Brenner, M. B., Trowbridge, I. & Strominger, J. L. *Cell* **40**, 183–190 (1985).
- Borst, J. *et al. Nature* **312**, 455–458 (1984).
- Weiss, A. & Stobo, J. D. *J. exp. Med.* **160**, 1284–1299 (1984).
- Ohashi, P. S. *et al. Nature* **316**, 606–609 (1985).
- Weiss, A. *et al. Rev. Immun.* **4**, 593–619 (1986).
- Heilig, J. S. *et al. Nature* **317**, 68–70 (1985).
- Zauderer, M., Iwamoto, A. & Mak, T. W. J. *exp. Med.* **163**, 1314–1318 (1986).
- Hayday, A. C. *et al. Cell* **40**, 259–269 (1985).
- Kranz, D. M. *et al. Nature* **313**, 752–755 (1985).
- Murre, C. *et al. Nature* **316**, 549–552 (1985).
- Le Franc, M.-P., Forster, A., Baer, R., Stinson, M. A. & Rabbitts, T. H. *Cell* **45**, 237–246 (1986).
- Lefranc, M.-P. & Rabbitts, T. H. *Nature* **316**, 464–466 (1985).
- Quertermous, T. *et al. Science* **231**, 252–255 (1986).
- Quertermous, T. *et al. Nature* **322**, 184–187 (1986).
- Dialynas, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 2619–2623 (1986).
- Snodgrass, H. R., Dembic, Z., Steinmetz, M. & Von Boehmer, H. *Nature* **315**, 232–233 (1985).

- Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103–107 (1985).
- Sangster, R. N. *et al. J. exp. Med.* **163**, 1491–1508 (1986).
- Brenner, M. B. *et al. Nature* **322**, 145–149 (1986).
- Bank, I. *et al. Nature* **322**, 179–181 (1986).
- Van de Griend, R. J. *et al. Clin. Immun. Immunopath.* **21**, 94–106 (1981).
- Van de Griend, R. J. *et al. Immunology* **47**, 313–320 (1982).
- De la Hera, A., Toribio, M. L., Marquez, C. & Martinez-A, C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6268–6271 (1985).
- Van de Griend, R. J. & Bolhuis, R. L. H. *Blood* **65**, 1002–1009 (1985).
- Spits, H. *et al. J. Immun.* **135**, 1922–1928 (1985).
- Lanier, L. L., Kipps, T. J. & Phillips, J. H. *J. exp. Med.* **162**, 2089–2106 (1985).
- Van de Griend, R. J., Giphart, M. J., Van Krimpen, B. A. & Bolhuis, R. L. H. *J. Immun.* **133**, 1222–1228 (1984).
- Van de Griend, R. J. *et al. J. Immun.* **138**, 1987.
- Spits, H., Yssel, H., Leeuwenberg, J. & De Vries, J. E. *Eur. J. Immun.* **15**, 88–91 (1985).
- Leeuwenberg, J. F. M., Spits, H., Tax, W. J. M. & Capel, P. J. A. *J. Immun.* **134**, 3770–3775 (1985).
- Bolhuis, R. L. H. & Van de Griend, R. J. *Cell Immun.* **93**, 46–57 (1985).
- Ritz, J. *et al. Science* **228**, 1540–1543 (1985).
- Lanier, L. L., Cwirla, S., Federspiel, N. & Phillips, J. H. *J. exp. Med.* **163**, 209–214 (1986).
- Quertermous, T. *et al. J. Immun.* (in the press).
- Greenberg, J. M., Quertermous, T., Seidman, J. G. & Kersey, J. H. *J. Immun.* **137**, 2043–2049 (1986).
- Van Dongen, *et al. J. Immun.* (in the press).
- Elder, J. H. & Alexander, S. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4540–4544 (1982).
- Nowill, A. *et al. J. exp. Med.* **163**, 1601–1606 (1986).
- Furley, A. J. *et al. Cell* **46**, 75–87 (1986).
- Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998–7002 (1986).
- Bolhuis, R. L. H., Gravekamp, C. & Van de Griend, R. J. *Clin. Immun. Allergy* **6**, 29–90 (1986).
- Tax, W. J. M., Willems, H. W., Reekers, P. P. M., Capel, P. J. A. & Koene, R. A. P. *Nature* **304**, 445–447 (1983).
- Oetting, H. C., Petter, C., Maloy, W. L. & Terhorst, C. *Nature* **320**, 272–275 (1986).
- O'Farrell, P. H. *J. biol. Chem.* **250**, 4007–4021 (1975).

Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes

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The T-cell receptor (TCR) γ polypeptide is expressed associated with CD3 (T3) on the surface of normal human peripheral blood lymphocytes. These cells function as non-MHC-restricted cytotoxic T lymphocytes (CTL) and thus may play an important role in host immune defence. The TCR γ polypeptide occurs as a dimer in at least two molecular forms based on the absence or presence of disulphide linkage. These forms use TCR γ polypeptides with strikingly different peptide backbone sizes.

In addition to the TCR α and β genes, a third gene, γ , that rearranges in T cells exists in mouse¹⁻³ and in man^{4,5}. This gene encodes a cell surface polypeptide of relative molecular mass 55,000 (M_r 55K) based on recognition by TCR γ -specific anti-peptide sera⁶. The TCR γ polypeptide was found with another polypeptide, δ (40K), physically associated with the CD3 (T3) glycoprotein on the cell surface⁶. TCR $\gamma\delta$ lymphocytes were found to be CD3⁺4⁻8⁻ (double negative) or CD3⁺4⁻8⁺ in the peripheral blood of immunodeficiency patients and on a thymic-derived T cell clone^{6,7}.

The distribution and the function of cells that express the TCR γ product remain an enigma. At present, two lines of evidence suggest divergent roles for cells expressing the TCR γ polypeptide. Because TCR γ messenger RNA is expressed before expression of TCR β or TCR α mRNA during fetal thymic ontogeny in the mouse, a direct role in thymic differentiation of TCR $\alpha\beta$ lymphocytes was suggested^{8,9}. But the TCR $\gamma\delta$ complex has been identified on human CD3⁺ immunodeficiency patient lymphocytes that failed to react with framework monoclonal antibodies (mAb) against the TCR $\alpha\beta$ complex (WT31 and β F1) and similarly failed to express mRNA encoded by

the known TCR α and β genes⁶. Moreover, cells of the same phenotype, namely WT31⁻ β F1⁻CD3⁺ were found in normal human peripheral blood^{6,10,11}. These results suggest that the second TCR might also occur on T lymphocytes that are part of the mature effector T cell repertoire.

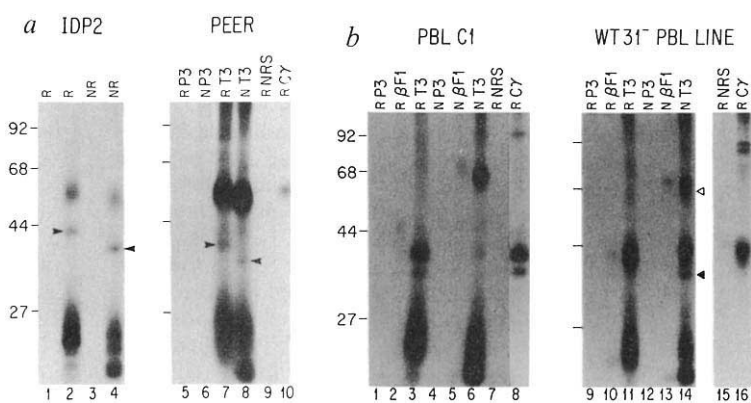
Here we show that the TCR γ polypeptide occurs in normal human peripheral blood on CD3⁺ non-major histocompatibility complex (MHC) restricted cytotoxic lymphocytes. Moreover, based upon the absence or presence of disulphide-linkage and peptide backbone size, CD3-associated TCR γ peptides are shown to exist in at least two molecular forms.

The non-disulphide-linked form

The immunodeficiency patient 2 (IDP2) cell line was sorted for WT31⁻ cells and has been carried continuously *in vitro* for more than one year. It is homogeneously WT31⁻ β F1⁻CD2⁺3⁺ (T11⁺T3⁺) and CD4⁻8⁻. Immunoprecipitations from lysates of surface-iodinated IDP2 lymphocytes using anti-CD3 mAb (under conditions that do not dissociate TCR subunits from CD3, see Fig. 1, legend) yielded two species (55K and 40K) in addition to the CD3 subunits (Fig. 1a). This result is identical

Fig. 1 Immunoprecipitations of TCR γ , δ and CD3 from a human tumour and peripheral blood lymphocyte lines. Immunoprecipitations from ¹²⁵I-labelled cell lysates were analysed by SDS-PAGE (10% acrylamide) under reducing (R) or nonreducing (N or NR) conditions. Size markers, M_r in thousands. **a**, TCR γ , δ and CD3 subunits on IDP2 and PEER cells. Immunoprecipitations were performed using 1 μ g control mAb P3 (mAb secreted by the P3X63.Ag8 myeloma lanes 1, 3, 5 and 6); 1 μ g UCHT1 (anti-CD3)²¹ (lanes 2, 4, 7 and 8); 10 μ l normal rabbit serum (NRS lane 9) and 10 μ l anti-C γ peptide sera (anti-TCR γ)⁶ (lane 10). Arrows, positions of TCR δ subunits which change mobility under R and NR conditions. **b**, TCR γ , δ and CD3 subunits on peripheral blood T-cell clone, PBL C1, and the WT31⁻PBL LINE. Immunoprecipitations were performed using control mAb P3 (lanes 1, 4, 9 and 12), 1 μ g β F1 (anti-TCR β)¹² (lanes 2, 5, 10 and 13), NRS (lanes 7 and 15) and anti-C γ peptide sera (lanes 8 and 16). Open arrow, disulphide-linked, β F1 unreactive CD3-associated species; solid arrow, non-disulphide-linked, CD3-associated material that displays increased SDS-PAGE mobility under nonreducing condition (like TCR δ in **a**).

Methods. Viable lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation and 2×10^7 cells were radio-iodinated by the lactoperoxidase technique as described²². Labelled cells were lysed in 5 ml of TBS (10 mM TRIS pH 8, 140 mM NaCl) with 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate (CHAPS), which preserves the TCR-CD3 association²³, containing 2 mM phenylmethylsulphonyl fluoride (PMSF) and 8 mM iodoacetamide (IAA). Immunoprecipitation was carried out using fixed *Staphylococcus aureus* Cowan I (SACI) as described²⁴, and the immune complexes were washed $\times 5$ in TBS containing 0.1% Triton X-100 (TX-100). Reduced samples were boiled in 2 mM dithiothreitol (DTT) and all samples incubated for 10 min at 23 °C in 10 mM IAA before analysis by SDS-PAGE. Immunoprecipitations using anti-C γ sera were performed on 1% TX-100 lysates that were dialysed to remove IAA and then denatured by the addition of $\frac{1}{10}$ vol of sodium dodecyl sulphate (SDS) containing 3 mM DTT with boiling for 3 min. After partial renaturation by the addition of 4 vols of 1.5% TX-100 in TBS containing 30 mM IAA, anti-C γ sera or NRS were added and the immunoprecipitates were washed in TBS containing 0.5% TX-100, 0.5% deoxycholate, 0.05% SDS before analysis by SDS-PAGE. Rat anti-mouse κ chain-specific mAb 187.1 (5 μ g) was added as a second antibody to provide protein A binding of IgG₁ mAb β F1, UCHT1 and P3²⁵.



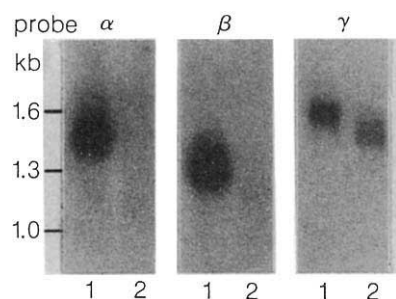


Fig. 2 Northern blot analysis of RNA isolated from PBL C1. Total RNA preparations from the T leukaemic cell line HPB-MLT (lane 1 for each probe) and PBL C1 (lane 2 for each probe) were analysed on Northern blots using TCR α , TCR β and TCR γ cDNA probes.

Methods. RNA preparation, agarose gel electrophoresis, transfer to nitrocellulose, hybridization with 32 P-labelled, nick-translated probes and autoradiography were as described⁶. Approximately 1.5 μ g RNA was loaded per lane, probes were labelled to similar specific activity, and identical autoradiographical exposures are presented. RNA sizes were determined based on previously published lengths for TCR β and TCR γ transcripts^{26,27}.

to the one reported previously using chemical cross-linking. The 55K species was shown to react specifically with anti- C_γ and anti- V_γ peptide sera⁶. The 40K polypeptide was unreactive with these anti- γ peptide sera and is thus likely to represent a non TCR α , β or γ subunit, designated δ^6 . To determine if these subunits are covalently linked, like the TCR α and β subunits, the CD3 co-immunoprecipitated polypeptides were examined under reducing and nonreducing conditions. In striking contrast to the TCR $\alpha\beta$ subunits, which exist in a heterodimeric disulphide-linked form under nonreducing conditions, the TCR γ and δ subunits on the IDP2 cell line are not covalently linked (Fig. 1a). A small increase in relative mobility on SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions was observed for the diffuse, heavily glycosylated (see below) TCR γ , whereas a dramatic increase in mobility was observed for the δ subunit, suggesting the presence of one or more intrachain disulphide loops (compare species at arrows, lanes 2 and 4).

Weiss *et al.* suggested that the PEER cell line might express the TCR γ polypeptide as it lacked expression of TCR α mRNA yet expressed a CD3-associated 55–60K polypeptide¹⁰. On further examination, this cell line was found to lack reactivity with a mAb recognizing framework determinants on the TCR β chain, β F1 (Fig. 1a) and to express a strongly iodinated 55–60K polypeptide associated with a weakly iodinated 38K polypeptide. The 55–60K polypeptide was specifically immunoprecipitated with anti- C_γ peptide sera and thus appears to represent a further example of the TCR γ protein (Fig. 1a). The TCR γ and δ polypeptides on PEER were of similar size to those on the IDP2 cell line and similarly were not disulphide-linked. Like the δ subunit on IDP2 cells, the counterpart molecule on PEER underwent a marked shift in SDS-PAGE mobility when compared under reducing and nonreducing conditions (compare species at arrows, lanes 7 and 8). Thus the IDP2 and the PEER cell lines appear to express similar types of TCR $\gamma\delta$ -CD3 complexes, in which the TCR γ and δ subunits are not covalently linked.

The disulphide-linked form

We wished to determine if this second TCR was also expressed as a component of the T cell population in normal peripheral blood. Two-colour cytofluorographic analysis comparing staining of human peripheral blood lymphocytes (PBL) with mAb β F1 and OKT3 (anti-CD3) showed a discrete population representing 2–5% of the CD3⁺ PBL that appeared to be TCR $\alpha\beta$ negative⁶. To examine this lymphocyte population, normal adult

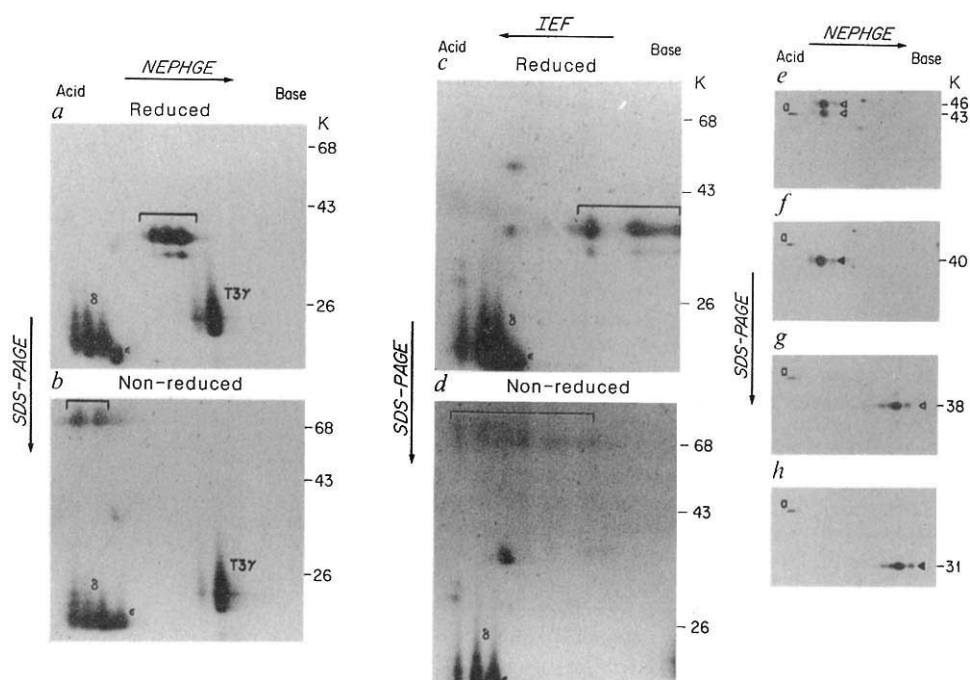
PBL were subjected to cytofluorographic cell sorting after staining with mAb WT31. Unstained PBL were isolated and propagated *in vitro* in IL-2-containing conditioned media receiving biweekly additions of irradiated autologous feeder cells and phytohaemagglutinin (PHA-P). The cell line derived, WT31⁺-PBL LINE, was cloned by limiting dilution with plating at 0.5 cell per well and the cloned cells were propagated as for the polyclonal cell line. Several such peripheral blood-derived T-cell clones were obtained, and PBL Clone 1 (PBL C1) was studied in detail. By cytofluorographic analysis, this clone was CD2⁺3⁺ but CD4[−]8[−] and WT31⁺. The expression of TCR α , β and γ mRNA from PBL C1 was determined by Northern blot analysis (Fig. 2). By comparison with the WT31⁺ β F1⁺ T-cell tumour HPB-MLT, only very low levels of TCR α and β mRNA were detected. In contrast, abundant TCR γ mRNA was noted (Fig. 2, γ probe); interestingly, the TCR γ mRNA was slightly smaller than the 1.6-kilobase (kb) message found in HPB-MLT and in the TCR γ -expressing IDP2 cell line (Fig. 2 and ref. 6). Consistent with these observations, WT31 reactivity was not detected in cytofluorographic analysis (data not shown) and only scant levels of TCR α and β polypeptides were found by immunoprecipitation using mAb β F1 (Fig. 1b, lanes 2, 5). It is likely that the trace levels of TCR α and β protein detected in PBL C1 are accounted for by the 1–2% contamination with irradiated autologous feeder cells used in the propagation of this clone. In contrast, two abundant chains (40K and 36K) were observed associated with CD3 under reducing conditions in SDS-PAGE analysis (Fig. 1b, lane 3). Anti- C_γ sera immunoprecipitated both of these polypeptides from reduced and denatured PBL C1 lysates (Fig. 1b, lane 8).

To determine if these 40K and 36K TCR γ polypeptides were part of a disulphide-linked dimer, co-immunoprecipitations with anti-CD3 were examined under nonreducing conditions. A single band of M_r 70K was observed indicating that, unlike IDP2 and PEER cells, PBL C1 expresses a CD3-associated TCR γ gene product that is part of a disulphide-linked dimeric complex.

As a TCR γ partner, δ , was present on the non-disulphide-linked form of this receptor complex on IDP2 and PEER cells, we examined whether the disulphide-linked form of the receptor on PBL C1 was composed of a homo- or a heterodimer. Immunoprecipitates were analysed by two-dimensional gel electrophoresis (nonequilibrium pH gel electrophoresis (NEPHGE) followed by SDS-PAGE; Fig. 3a, b). Under reducing conditions, both the TCR γ species (40K and 36K) were found to have identical charges, and displayed heterogeneity typical for a sialylated glycoprotein. These characteristics are like those described previously for differentially glycosylated TCR α polypeptides having the same amino-acid backbone¹². Thus, these species may represent differentially glycosylated forms of the same TCR γ peptide. This conclusion is supported by the results of metabolic pulse labelling (below) which reveal only a single precursor TCR γ species in PBL C1.

A disulphide-linked dimer composed of one or both of these TCR γ species should have a focusing position similar to either of the two components alone when analysed by NEPHGE or equilibrium isoelectric focusing (IEF). But a heterodimer composed of TCR γ and a distinct polypeptide might have a different charge and focusing position. The position of the disulphide-linked dimer was therefore examined by carrying out NEPHGE under nonreducing conditions, followed by SDS-PAGE under nonreducing conditions (Fig. 3b). Strikingly, the position of the disulphide-linked dimer was substantially more acidic than that of the TCR γ polypeptides examined under reducing conditions (compare the 40K and 36K species in Fig. 3a with the 70K species in Fig. 3b). This result suggests that the TCR γ species were covalently linked to a polypeptide of distinct NEPHGE mobility. Thus, although a TCR γ partner could not be directly visualized (either because it was inadequately labelled with 125 I or because it did not resolve in the focusing system used here),

Fig. 3 Two-dimensional gel analysis of TCR γ polypeptides and precursors. *a-d*, Comparison of reduced (separated) and nonreduced (dimeric) CD3-associated polypeptides from PBL C1. 125 I-labelled PBL C1 cells were lysed in CHAPS and immunoprecipitated with anti-CD3 mAb. Two-dimensional gel electrophoresis was carried out under reducing conditions (*a, c*) or nonreducing conditions (*b, d*). The CD3 γ , δ and ϵ positions are labelled and focused to similar positions under both R and N conditions. After cleaving the disulphide bond, the CD3-associated polypeptides (40K and 36K) migrated to focusing positions close to CD3 γ under R conditions, but shifted to a more acidic position (close to CD3 δ) under N conditions (when both components of the dimer were present (68K)). T3 γ represents CD3 γ . Size markers, M_r . *e-h*, Analysis of glycosylated and nonglycosylated IDP2 and PBL C1 TCR γ peptide precursors. IDP2 and PBL C1 cells were pulse-labelled with 35 S-methionine,



lysed under denaturing conditions and immunoprecipitated with anti- C_γ peptide sera. Immunoprecipitations were then either treated with endo-H or mock treated and analysed by two-dimensional gel electrophoresis. Glycosylated TCR γ peptides are denoted by open arrows, and nonglycosylated TCR γ peptides by solid arrows. Apparent M_r s were calculated from migration of standards used in panels *a* and *b* (not shown). A small amount of contaminating actin, denoted by a diamond in each panel, served as an internal marker. *e*, IDP2 TCR γ , mock incubated; *f*, IDP2 TCR γ , endo-H treated; *g*, PBL C1 TCR γ mock incubated; *h*, PBL C1 TCR γ endo-H treated.

Methods. *a-d*, After radioiodination with lactoperoxidase, lymphocytes were treated with 100 U of neuraminidase (Gibco) in phosphate-buffered saline (PBS), 1 mg ml $^{-1}$ bovine serum albumin, 1 mg ml $^{-1}$ glucose for 90 min at 23 °C, washed in PBS and solubilized in 0.3% CHAPS. Immunoprecipitates were prepared as in Fig. 1 and NEPHGE (charge separation) was carried out using pH 3.5–10 ampholines (LKB, Sweden), or IEF using pH 3.5–10, 4–6, 9–11 ampholines (2:1.5:1.5) followed by 10.5% SDS-PAGE gels for size separation as described¹². NEPHGE was carried out (*a, b*) applying the iodinated sample at the acidic end; IEF (*c, d*) was carried out for 20 h at 400 V, applying the sample at the other (basic) end. Brackets, CD3-associated species. *e-h*, Cells (2×10^7) were preincubated for 1 h at 37 °C in 4 ml methionine-free RPMI 1640 supplemented with 10% fetal bovine serum. 35 S-methionine was added to 250 μ Ci ml $^{-1}$, and incubation was continued for 1 h at 37 °C. Cells were collected, washed and lysed in 0.4 ml of a boiling solution of 1% SDS, 10 mM Tris-HCl (pH 8.0), 0.1 mM PMSF and 10 mM IAA. Lysates were diluted with 1.6 ml of 2.5% Nonidet-P40, 1% gelatin, 10 mM Tris-HCl (pH 8), and 0.2 ml of 1 mg ml $^{-1}$ DNase, 0.5 mg ml $^{-1}$ RNase, 0.5 M Tris-HCl (pH 7), 50 mM MgCl $_2$, and incubated at 0 °C for 2–4 h. After centrifugation for 15 min at 12,000g to pellet insoluble debris, immunoprecipitations with anti- C_γ serum were performed using protein A Sepharose preincubated with 1% gelatin and washed as described²⁸. Elution from the immunoabsorbent and treatment with endo-H (Miles Scientific) were as described²⁸. Samples were analysed by two-dimensional gel electrophoresis employing NEPHGE in the first dimension and 10% SDS-PAGE in the second dimension, followed by fluorography²⁹.

the TCR γ polypeptide on PBL C1 appeared to be expressed as a part of a disulphide-linked heterodimer. Experiments using equilibrium IEF (rather than NEPHGE) confirmed this observation (Fig. 3*c,d*).

Different TCR γ peptide backbones

A further distinction between the disulphide-linked and non-linked forms was the size of the mature TCR γ glycopeptide (55–60K on IDP2 and PEER versus 40K and 36K on PBL C1). To assess how much of this radical size difference is due to differential glycosylation and how much to different peptide backbones, TCR γ peptides were analysed in cells pulse-labelled with 35 S-methionine. After solubilization under denaturing and reducing conditions, the lysates were immunoprecipitated with anti- C_γ sera and examined by two-dimensional gel electrophoresis (Fig. 3*e-h*). Immunoprecipitates were either treated with endoglycosidase H (endo-H) to remove immature high-mannose glycans from pulse-labelled material, or were mock treated. Two TCR γ polypeptides (46K and 43K) of identical NEPHGE mobility were synthesized by the IDP2 cell line. Treatment with endo-H reduced both forms to a 40K form, suggesting that the 46K and 43K forms carried different numbers of carbohydrates, and that a single TCR γ polypeptide backbone (40K) was synthesized by IDP2 cells (Fig. 3*e,f*). In contrast, a more basic, 38K glycosylated form was synthesized by PBL C1, which after endo-H digestion displayed a nonglycosylated 31K

peptide backbone (Fig. 3*g, h*). Thus, the TCR γ polypeptides on the non-disulphide-linked (IDP2) and the disulphide-linked (PBL C1) forms characterized here have radically different peptide backbone sizes (40K and 31K, respectively). The fact that the glycosylated TCR γ peptides observed by pulse-labelling are of different molecular weight from those found by cell surface iodination presumably results from the different types of carbohydrates they carry, namely high-mannose versus complex.

Two forms of TCR γ in peripheral blood

We next wished to determine if both a disulphide-linked and a noncovalently associated form occurred in normal adult peripheral blood. The polyclonal peripheral blood cell line (WT31 $^-$ PBL LINE) from which PBL C1 had been cloned was therefore studied in greater detail. WT31 $^-$ PBL LINE was homogeneously CD2 $^+$ 3 $^+$ and contained 95% WT31 $^-$ CD4 $^-$ 8 $^-$ with 5% contaminating WT31 $^+$ cells. When examined by immunoprecipitation from iodinated, solubilized cells, weak but detectable reactivity with mAb β F1 was observed (Fig. 1*b*, lanes 10 reduced and 13 nonreduced), consistent with the expected 5% TCR $\alpha\beta$ positive lymphocytes. In contrast, anti-CD3 mAb immunoprecipitated large amounts of both CD3 and associated polypeptides of 35–45K under reducing conditions (Fig. 1*b*, lane 11). To determine what fraction of these were disulphide-linked, the CD3 immunoprecipitate was examined under non-reducing conditions (Fig. 1*b*, lane 14). Less than half of the

CD3-associated polypeptides were disulphide-linked. This material included disulphide-linked TCR $\alpha\beta$ peptides located above the open arrow, lane 14 (size identified by the β F1 precipitate, lane 13) and disulphide-linked TCR γ peptides of smaller size (open arrow, lane 14). Strikingly, the majority of the CD3-associated species were not disulphide-linked and migrated with the same mobility under both reducing and nonreducing conditions. Notably, a fraction of these non-linked species displayed a marked increase in SDS-PAGE mobility under nonreducing conditions, similar to the TCR δ on the IDP2 and PEER cells (see Fig. 1b, lane 14, solid arrow). Reactivity with anti- C_γ sera confirmed that most of the labelled material associated with CD3 expressed on WT31⁺PBL LINE was TCR γ gene product (lane 16).

Thus, the protein product of the TCR γ gene occurs on CD3⁺ lymphocytes in adult peripheral blood in both disulphide-linked and unlinked molecular forms. Moreover, the non-disulphide-linked form of TCR γ may be further divided into 55–60K glycosylated (IDP2 and PEER) or 35–45K glycosylated (thymic T-cell clone CII⁷ and WT31⁺PBL LINE) species.

TCR γ rearrangements

TCR γ and β gene rearrangements were examined in T cells known to express the TCR γ polypeptide on their cell surfaces. Southern blot analyses were carried out using the 0.8-kb *EcoRI*–*HindIII* human $J_{\gamma 1.3}$ probe (nomenclature according to Quertermous *et al.*¹³). This probe detects germline bands of 23 kb and 12 kb in a *Bam*HI digest of genomic DNA. The 23-kb band encompasses $C_{\gamma 1}$, and the 12-kb band encodes $C_{\gamma 2}$. Using this probe, IDP2, PBL C1 and PBL C2 (also derived from the WT31⁺PBL LINE) showed rearrangements of the TCR γ gene (both PBL C1 and PBL C2 displayed an identical rearrangement; Fig. 4a).

Seven rearrangements in PBL using the $J_{\gamma 1.3}$ probe and *EcoRI*-digested genomic DNA in Southern blot analyses have been detected¹⁴. Six (I, II, III, IV, VII and V) of these seven rearrangements are shown in PBL, fetal thymus, and newborn thymus genomic DNA (Fig. 4b; see arrows and rearrangement numbers). Four rearrangements (numbers I, II, VI and VII) either are not used by peripheral blood lymphocytes which express the TCR γ polypeptide or cells demonstrating them were lost under the propagation conditions used for the WT31⁺PBL LINE. Nevertheless, the WT31⁺PBL LINE DNA revealed at least three of these rearrangements (III, IV and V) (Fig. 4b, lane 3) and these same rearrangements were used by IDP2, PBL C1 and PBL C2 (data not shown for the *EcoRI* digest) and all of these rearrangements are displayed in fetal thymus.

The TCR β gene was also rearranged in IDP2, PBL C1 and PBL C2 cells. The 1.1-kb *EcoRI*–*HindIII* $C_{\beta 2}$ probe detects a germline band of 20 kb which encompasses both C_β constant regions in a *Bam*HI digest of genomic DNA¹⁵. One predominant TCR β rearrangement for IDP2 and two identical rearrangements for PBL C1 and PBL C2 were observed (Fig. 4c). It is assumed that these TCR β rearrangements are nonproductive based on the immunoprecipitations and Northern analyses for these cell lines. As both PBL C1 and PBL C2 have the same TCR γ and β rearrangements, they appear to be clonal and derived from the same cell within the WT31⁺PBL LINE.

Non-MHC restricted cytotoxic lymphocytes

As TCR γ -expressing cells were found in adult peripheral blood, functional studies were carried out to determine whether they have effector capabilities. When IDP2 and PBL C1 were examined for their ability to lyse target cells in ⁵¹Cr release assays, they proved to have spontaneous effector cytotoxic capability (Fig. 5). Although the IDP2 cell line did not lyse the majority of natural killer (NK) targets or PHA blasts of allogeneic PBL, they were selectively capable of lysing ⁵¹Cr-labelled MOLT-4 cells (Fig. 5a, top). In two of six similar assays, weak lysis (10–15% ⁵¹Cr release) of K562 targets was also

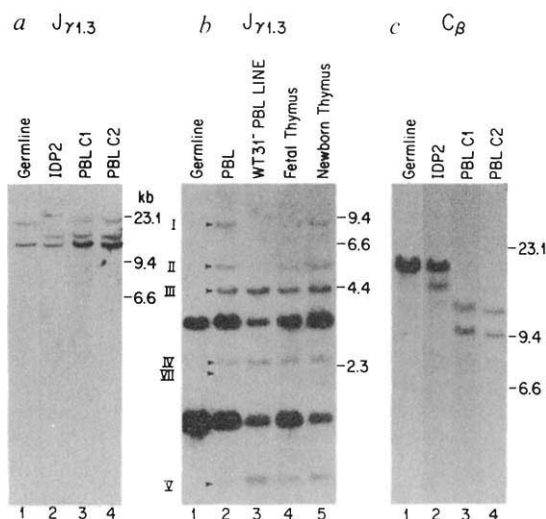


Fig. 4 Rearrangements of the γ and β genes in T cells expressing the TCR γ polypeptide. Genomic DNAs isolated from the IDP2 cell line, PBL C1, PBL C2, WT31⁺PBL LINE, fetal thymus, newborn thymus, PBL and a B cell line (JY for germline) were examined in Southern blot analysis for TCR γ (a, b) and TCR β (c) gene rearrangements. Genomic DNAs were digested with *Bam*HI (a, c) or *EcoRI* (b), fractionated on agarose gels and transferred to nitrocellulose filters for hybridization with ³²P-labelled $J_{\gamma 1.3}$ (a, b) or $C_{\beta 2}$ probes (c). Arrows and roman numerals denote TCR γ rearrangements. Size markers in kb.

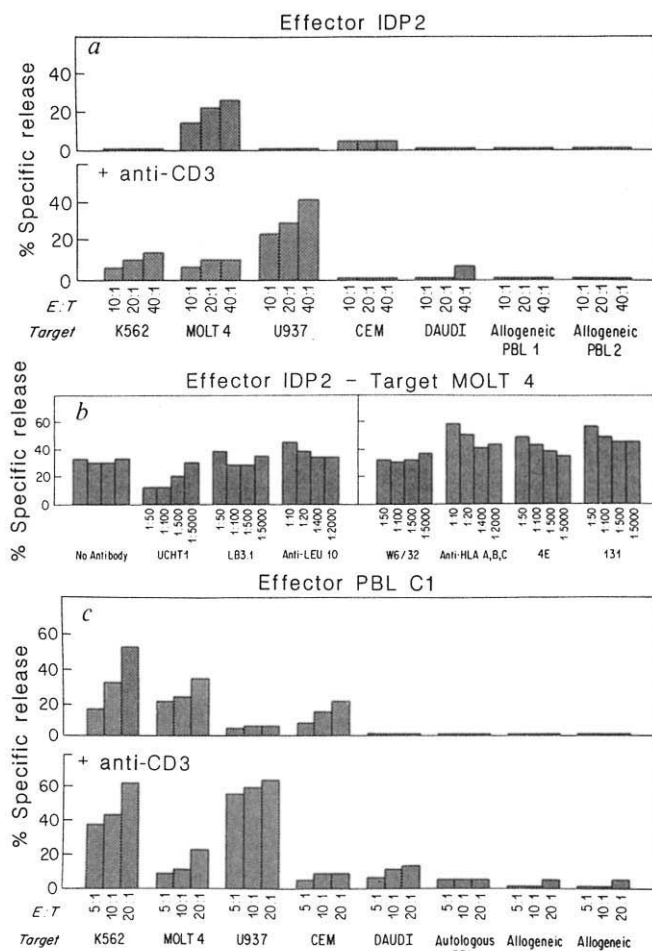
Methods. Genomic DNA was isolated as described³⁰, digested with endonuclease (*Bam*HI or *EcoRI*), size-fractionated on 0.7% agarose (*Bam*HI digests) or 0.9% agarose (*EcoRI* digest), and transferred to nitrocellulose as described³¹. Filters were hybridized to a nick-translated ³²P-labelled 0.8-kb *HindIII*–*EcoRI* $J_{\gamma 1.3}$ probe (ref. 14 and unpublished) or a 1.1-kb *EcoRI*–*HindIII* $C_{\beta 2}$ probe¹⁵. Filters were washed in 2×SSC and 0.1% SDS followed by 0.2% ×SSC and 0.1% SDS at 55 °C before autoradiography with intensifying screens.

observed. Lysis of MOLT-4 cells was not inhibited by a variety of mAbs directed against monomorphic MHC Class I (W6/32, anti-HLA-A, B, C, 4E and 131) or Class II (LB3.1 and anti-Leu 10) determinants (Fig. 5b), although we have previously found that these mAbs efficiently block killing by both MHC Class I and Class II allospecific CTL¹⁶. These data suggest that lysis of MOLT-4 cells was MHC class I and II independent. Only anti-CD3 mAb partially blocked the specific lysis of MOLT-4 cells (Fig. 5b). On the other hand, when triggered by prebinding of anti-CD3 mAb to IDP2, as has been previously reported for thymic-derived CII⁷, ⁵¹Cr-labelled target cells that express Fc receptors for IgG (for example, U937), were efficiently lysed (Fig. 5a, +anti-CD3). Such killing could be completely inhibited by aggregated human IgG, confirming that this CD3-mediated lysis occurred through a mechanism of enhanced conjugate formation via IgG Fc receptors (data not shown). The paradoxical augmentation of lysis by anti-CD3 mAb for some targets (U937) and the blocking of lysis for specifically recognized targets (MOLT-4) might result from the competing effects of triggering and increasing conjugate formation but sterically blocking antigen recognition via the TCR.

PBL C1 proved a more efficient killer cell than IDP2. PBL C1 displayed spontaneous cytolytic activity against K562 cells (MHC Class I and II negative) showing nearly 50% specific ⁵¹Cr release when examined at an effector:target (E:T) ratio of 20:1 (Fig. 5c, top). Moreover, PBL C1 also lysed MOLT-4 cells and to a lesser extent, CEM cells. No lysis of Daudi, U937, or either autologous or allogeneic PBL was detected. Triggering with anti-CD3 mAb induced PBL C1 to lyse the U937 cell line. Further, lysis of K562 was slightly augmented whereas that of MOLT-4 was partially inhibited (Fig. 5c). Taken together, the spontaneous cytolytic activity of IDP2 and PBL C1 on tumour

Fig. 5 Cytolysis by IDP2 and PBL C1 cells. *a, c*, IDP2 or PBL C1 effector cells were incubated (at effector:target (E:T) ratios indicated) with ^{51}Cr -labelled target cells K562 (erythroid line), U937 (monocytic line), MOLT-4, CEM (T leukaemic lines), Daudi (Burkitt's lymphoma line) or allogeneic or autologous PBL (3-day PHA blasts of human PBL). The % specific release of ^{51}Cr for each target is shown. The same assays were carried out after prebinding anti-CD3 mAb UCHT1 to the effector cells for 30 min at 0°C (+ anti-CD3). *b*, Inhibition of IDP2 cytolysis of MOLT-4 target cells by various mAbs. IDP2 and ^{51}Cr -labelled MOLT-4 cells were incubated together at a 40:1 E:T ratio in the presence of various dilutions of anti-MHC Class I mAb W6/32 (anti-HLA-A, B, C monomorphic determinant)³², anti-HLA-A, B, C (monomorphic determinant)³³, 4E (anti-HLA-B and C locus)³⁴, 131 (anti-HLA-A locus)³⁵ or anti-MHC Class II mAb LB3.1 (anti-DR specific)³⁶, anti-Leu 10 (anti-DQ specific)³⁷ or anti-CD3 mAb UCHT1. Higher dilutions were used for mAb available as ascites (W6/32, 4E, 131, LB3.1, and UCHT1) and lower dilutions were used for commercial mAb (anti-Leu 10) or culture supernatant (anti-HLA-A, B, C).

Methods. Cytolytic assays were performed in round-bottom 96-well tissue culture plates with ^{51}Cr -labelling, harvesting and calculation of % specific release as described¹⁶. For *a* and *c*, IDP2 or PBL C1 cells were either preincubated with UCHT1 (1:300 dilution) (+ anti-CD3) for 30 min at 0°C , washed $\times 3$ or mock incubated and placed together with labelled target cells. Anti-HLA Class I and Class II mAb or anti-CD3 mAb were placed in wells containing the ^{51}Cr -labelled MOLT 4 cells for 30 min at 0°C , then IDP2 cells were added at a 40:1 E:T ratio. All samples were assayed in triplicate, each experiment was performed at least three times, and one representative experiment of each is shown.



targets such as K562 and MOLT-4 and the failure to block such activity by anti-MHC mAb suggests these TCR γ lymphocytes are non-MHC class I- or class II-restricted CTL.

Discussion

TCR γ (55K) and δ (40K) have previously been identified as CD3-associated peptides on immunodeficiency patient cells. Here, these two subunits are shown to be noncovalently associated. Importantly, the TCR γ polypeptide is also shown to be part of a second TCR that occurs on normal adult peripheral blood lymphocytes. A T-cell clone isolated from peripheral blood (PBL C1), expresses two peptides of 40K and 36K that specifically react with anti-C γ peptide sera. In contrast to the IDP2 cell line, these TCR γ polypeptides on PBL C1 are of smaller size and are part of a disulphide-linked complex. This disulphide-linked complex appears to be a heterodimer as the focusing mobility of the dimer was drastically different from that of the TCR γ polypeptide. The TCR γ polypeptides from the disulphide-linked and non-linked forms not only have different glycosylated sizes (55-60K versus 36-40K) but also have different peptide backbone sizes (40K versus 31K, respectively) as well as different mRNA transcript lengths. Moreover, analysis of a PBL-derived polyclonal population (WT31⁻PBL LINE) which was composed predominantly of TCR γ lymphocytes revealed both disulphide-linked and non-linked TCR γ complexes. Interestingly, the non-disulphide-linked TCR γ subunits observed on PBL were of smaller glycosylated size (35-45K) than the non-disulphide-linked TCR γ species on the IDP2 and PEER cell lines (55-60K). Thus, TCR γ exists on the cell surface in at least two molecular forms (disulphide-linked and non-linked) and as two drastically different polypeptide sizes (glycosylated 55-60K and 36-40K). Both TCR glycopeptide sizes have been observed in non-disulphide-linked forms,

whereas only the smaller size has been observed in the disulphide-linked form.

The TCR γ lymphocytes examined here exhibit non-MHC-restricted cytolytic activity and may be similar to other CD3⁺ NK-like cells whose T-cell receptors have not yet been definitively characterized¹⁷⁻²⁰. As CTL they may participate in host immune surveillance against malignancy or infection. The specificity of lysis observed suggests the possibility of TCR γ mediated antigen-specific recognition of some but not all tumour targets. As anti-CD3 mAb could trigger nonspecific lysis of some target cells or alternatively block specific lysis of other targets, the CD3 molecule on these cells appears to be functional. This conclusion has been confirmed by the observation that anti-CD3 mAbs are capable of triggering abrupt changes in the levels of intracellular free calcium³⁸.

Based on the sequential expression of TCR γ , β and α mRNA during fetal thymic development, a developmental role for the TCR γ in the ontogeny of TCR $\alpha\beta$ cells has been suggested^{8,9}. The data reported here suggest that cells that have productively rearranged and expressed the TCR γ protein on their cell surfaces are mature cells found in peripheral blood. These cells use several of the TCR γ gene rearrangements that can be detected in fetal thymus. The relationship of TCR γ rearrangements in peripheral blood and thymus is consistent with the possibility that TCR γ cells pass through a stage of differentiation in the thymus. In this case, T cells that productively rearrange the TCR γ gene and express TCR γ at the cell surface may exit to the periphery as mature T cells and those that do not (either because TCR γ is nonproductively rearranged or because the TCR γ partner is not expressed) may then go on to rearrange and express the TCR $\alpha\beta$.

Use of a functional CD3 molecule and a TCR polypeptide encoded by a rearranging gene suggests that, like TCR $\alpha\beta$

lymphocytes, TCR γ lymphocytes may be capable of antigen-specific recognition and clonal expansion. As these cytotoxic T cells are found in peripheral blood, lymphocytes expressing the TCR γ polypeptide must be considered as part of the mature repertoire of functional T cells.

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1. Saito, H. *et al.* *Nature* **309**, 757-762 (1984).
2. Kranz, D. M. *et al.* *Nature* **313**, 752-755 (1985).
3. Hayday, A. C. *et al.* *Cell* **40**, 259-269 (1985).
4. Lefranc, M.-P. & Rabbitts, T. H. *Nature* **316**, 464-466 (1985).
5. Murre, C. *et al.* *Nature* **316**, 549-552 (1985).
6. Brenner, M. B. *et al.* *Nature* **322**, 145-149 (1986).
7. Bank, I. *et al.* *Nature* **322**, 179-181 (1986).
8. Raullet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103-107 (1985).
9. Snodgrass, H. R., Dembic, Z., Steinmetz, M. & von Boehmer, H. *Nature* **315**, 232-233 (1985).
10. Weiss, A., Newton, M. & Crommie, D. *Proc. natn. Acad. Sci. U.S.A.* **83**, 6998-7002 (1986).
11. Lanier, L. L. & Weiss, A. *Nature* **324**, 268-270 (1986).
12. Brenner, M. B. *et al.* *J. Immun.* (in the press).
13. Quertermous, T., Strauss, W. M., van Dongen, J. M. & Seidman, J. G. *J. Immun.* (in the press).
14. Quertermous, T. *et al.* *Science* **231**, 252-255 (1986).
15. Duby, A. D. & Seidman, J. G. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4890-4894 (1986).
16. Brenner, M. B. *et al.* *J. Immun.* **135**, 384-390 (1985).
17. De la Hera, A., Toribio, M. L., Marquez, C. & Martinez-A., C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6268-6271 (1985).
18. Nowill, A. *et al.* *J. exp. Med.* **163**, 1601-1606 (1986).
19. Lanier, L. L., Ruitenberg, J. J. & Phillips, J. H. *J. exp. Med.* **164**, 339-344 (1986).

20. Moingeon, P. *et al.* *Nature* **323**, 638-640 (1986).
21. Beverley, P. C. & Callard, R. E. *Eur. J. Immun.* **11**, 329-334 (1981).
22. Brenner, M. B., Trowbridge, I. S., McLean, J. & Strominger, J. L. *J. exp. Med.* **160**, 541-551 (1984).
23. Samelson, L. E., Harford, J., Schwartz, R. H. & Klausner, R. D. *Proc. natn. Acad. Sci. U.S.A.* **82**, 1969-1973 (1985).
24. Brenner, M. B., Trowbridge, I. S. & Strominger, J. L. *Cell* **40**, 183-190 (1985).
25. Yelton, D. E., Desaymard, C. & Scharff, M. D. *Hybridoma* **1**, 5-11 (1981).
26. Dyalynas, D. P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **83**, 2619-2623 (1986).
27. Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S. P., Aleksander, I. & Mak, T. W. *Nature* **308**, 145-149 (1984).
28. Krangel, M. S., Orr, H. T. & Strominger, J. L. *Cell* **18**, 979-991 (1979).
29. Bonner, W. J. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).
30. Wigler, M. *et al.* *Cell* **16**, 777-785 (1979).
31. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
32. Barnstable, C. J. *et al.* *Cell* **14**, 9-20 (1978).
33. Bushkin, Y., Posnett, D. N., Pernis, B. & Wang, C. Y. *J. exp. Med.* **164**, 458-473 (1986).
34. Yang, S. Y. *et al.* *Immunogenetics* **19**, 217-231 (1984).
35. Spear, B. T., Kornbluth, J., Strominger, J. L. & Wilson, D. B. *J. exp. Med.* **162**, 1802-1810 (1985).
36. Gorga, J. C., Foran, J., Burakoff, S. J. & Strominger, J. L. *Meth. Enzym.* **108**, 607-613 (1984).
37. Chen, Y. X. *et al.* *Hum. Immun.* **10**, 221-235 (1984).
38. Krangel, M. S. *et al.* *Proc. natn. Acad. Sci.* (in the press).

LETTERS TO NATURE

Low-frequency divergent X-ray variability in the Seyfert galaxy NGC4051

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The X-ray emission from NGC4051, a nearby low luminosity Type 1 Seyfert galaxy, exhibits variations that are both rapid and apparently quasi-periodic^{1,2}. Many Seyfert galaxies are variable³ but our knowledge of the form of variability has not advanced substantially since early well-resolved observations⁴ and attempts at statistical description⁵. Here we report an uninterrupted 62-h observation of NGC4051 with the EXOSAT observatory, giving an order of magnitude improvement in the quality of the time series, allowing comparison with X-ray binaries. The observed power spectral density is roughly proportional to $1/f$, similar to Cyg X-1, and characteristic of turbulent processes. No preferred timescale or luminosity gradient has been seen so far. If this behaviour holds generally for active galactic nuclei, quantities derived from short samples will be subject to selection effects. Some naive interpretations of variability are clearly invalid. Similar conclusions have recently been arrived at by McHardy *et al.* (see accompanying paper⁶).

NGC4051 was observed by EXOSAT for 62 h in December 1985, achieving a three- σ detection approximately every 50 s. Figure 1 shows the X-ray light curve, summed in 1,000-s bins. Variability is chaotic, and visually reminiscent of Cygnus X-1⁷. The flux ranges over a factor of 10, including a protracted episode of very low flux at the end of the observation. Figure 2 shows the power spectrum, calculated from the data summed in 50-s bins. At high frequencies ($>2 \times 10^{-3}$ Hz) the power spectrum is flat, variance being dominated by the Poisson noise of the data (indicated by a horizontal line in Fig. 2). At low frequencies (in the range 2×10^{-5} to 2×10^{-3} Hz) the spectrum

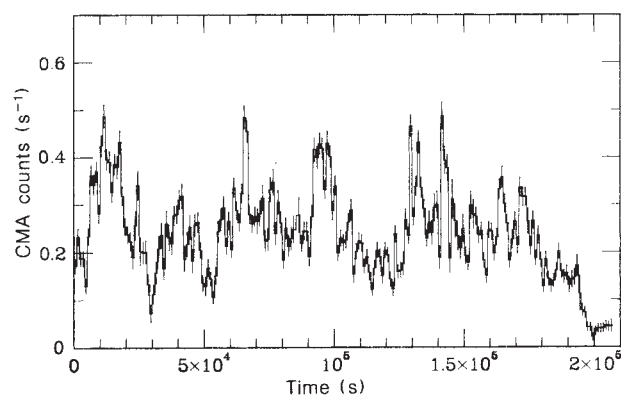


Fig. 1 X-ray light curve of NGC4051, commencing 1985 December 3 02:20 UT. Data presented here are from the EXOSAT¹⁸ low-energy energy imaging system, the Channel Multiplier Array (CMA, ref. 19), using the thin Lexan filter, covering the energy range 0.05–2 keV. The source was also seen in the medium energy (ME, ref. 20) proportional counter array, showing variability closely similar to that seen here with the CMA. Bin size used is 1,000 s, error bars are $\pm\sigma$. Data were background-subtracted and exposure-corrected as in².

is smooth and divergent at low frequencies, following a form close to $1/f$, where f is frequency. The shape is not necessarily exactly $1/f$, but for our purposes the important factors are the low-frequency divergence, and the fact that no preferred timescale stands out. We also cannot reject the possibility of modest curvature, but over the two decades which we can confidently measure, there is no substantial low-frequency flattening, or high-frequency steepening. The autocorrelation function (ACF) is consistent with our earlier measurement² at short lags, but does not confirm the 'zero-crossing time' of $\sim 3,000$ s, corresponding to the possible quasi-periodicity. The 3,000-s timescale is genuinely present in the earlier data, but our new data show that it is not characteristic of the underlying process. This discrepancy is typical of the problems that arise in short observations of low-frequency noise—structure always seems to appear on a timescale a few times shorter than the observation length.

The result is spectacular, but also frustrating. Low-frequency divergent 'flicker noise' is common in a wide range of circumstances⁸⁻¹¹ and its cause is unknown. We may conclude that the

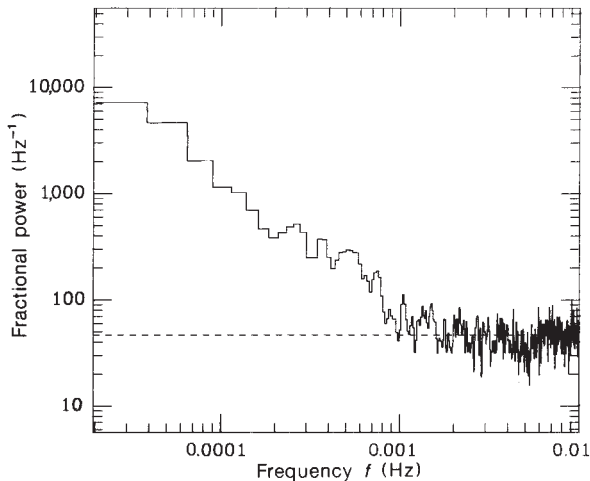


Fig. 2 Power spectral density, calculated by forming the auto-variance function (ACV), applying a Hanning roll-off, and Fourier transforming. The ACV was calculated from data summed in 50-s bins (4,531 samples), and was truncated at a lag of 512 bins, so that the lowest frequency represented in the power spectrum is sampled ~ 5 times. The power per Hz has been divided by the total sample variance, calculated using the same bin size, to give fractional contribution per Hz to the total observed variance. This includes contributions due to both experimental error (that is, counting statistics) and to real source variability.

X-ray emitting region is in a turbulent condition. There are also problems associated with the interpretation of variability, many of these arising from the pathological properties of $1/f$ noise⁹. We stress, however, that these do not depend on the spectral density exactly following $1/f$ over all frequencies, but only on its approximating $1/f$ in the observed regime. For a spectrum following $1/f^\alpha$, the power integrated to low frequencies diverges if $\alpha > 1$, and power integrated to high frequencies diverges if $\alpha < 1$. When $\alpha = 1.0$, power diverges at both ends. If the average logarithmic slope is close to 1, our sample estimates of total variance, mean and instantaneous values can be wrong by an unknown amount. Likewise, the local derivative of the time series diverges as one decreases the time step size, unless $\alpha > 2$.

Any timescale derived from such a light curve cannot be interpreted as a cooling timescale, synchrotron decay time, or similar, in order to constrain the physical properties of the emitting gas. All such effects give an exponentially decaying response to impulses, producing a power spectrum which is flat at low frequencies, and varies as $1/f^2$ at high frequencies⁷. (The logarithmic slope is equal to 1.0 at the knee frequency $f = 1/(2\pi\tau)$, where τ is the characteristic decay time, but the curvature is fast. A factor two above and below the knee frequency, the slope becomes 1.6 and 0.4, respectively, whereas the slope we see is close to 1 over two decades.) This means that models where variability is dominated by heating or cooling, for example, the Compton cooled hot cloud model for Cyg X-1¹², cannot explain $1/f$ variations.

Shot-noise models with exponential pulses can likewise be rejected. Shot noise can duplicate a $1/f$ spectrum if the shots are of a suitable shape (decaying as the square-root of time), or if the shots are exponential but have a range of decay times, with distribution function $D(\tau) \sim 1/\tau$. Similarly, heating-cooling models can be rescued by postulating a range of timescales. However such formal solutions are arbitrary unless the formalism is derived from a physical model.

There is no preferred timescale. The frequently used 'doubling timescale' is not well defined. For example, at the beginning of the observation, there is a decline by a factor of four over $\sim 20,000$ s, but towards the end of this, a sharp rise by a factor of two in $\sim 1,000$ s. In an observation of worse signal-to-noise, only the slow decay would be seen. The 'fastest doubling time-

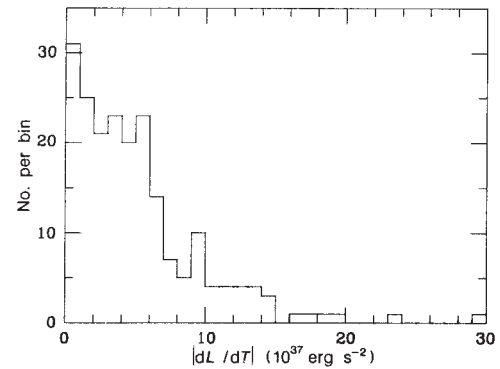


Fig. 3 Distribution of absolute values of dL/dt , in units of $10^{37} \text{ erg s}^{-2}$, calculated with 1,000-s bins.

scale' as used by Barr and Mushotzky⁵ thus depends on data quality and coverage. (Given an instrumental time resolution limit, one only has to wait long enough before seeing a factor of two change.) Furthermore, only for exponential events will the doubling timescale deduced from large and small amplitude changes be identical.

The shape of the power spectrum also implies problems for another quantity of interest, dL/dt , the rate of change of luminosity, which can be used in model-independent formulae to derive the efficiency of energy generation, and the compactness parameter¹³. The arguments used are weak if applied to individual fluctuations, so one should rather look for a characteristic value of $|dL/dt|$. Unfortunately, within the $1/f$ regime, the derivative diverges towards smaller timescales. So here again, the result found for dL/dt depends on data quality. (We confirmed this numerically, by making dL/dt light curves for various time resolutions, and comparing the dL/dt distributions.) Even for a given time resolution, it is not clear what quantity should be considered a 'characteristic' amplitude for dL/dt . The distribution of values of dL/dt is symmetric about zero, indicating no preference for rises to be faster than decays. The distribution of $|dL/dt|$ has a low modal value, but with a long tail of fluctuations many times larger than the mode. Figure 3 shows the distribution of $|dL/dt|$, using the data in 1,000-s bins. It is possible to derive a lower limit, but the problem of what to take as typical remains. At a resolution of 50 s, the largest values of dL/dt that we see are of the order of $10^{40} \text{ erg s}^{-2}$ (luminosity expressed in the range 0.1–1.0 keV, assuming spectrum as in ref. 2). A related quantity often used to define an 'objective' timescale is $L/(dL/dt)$. For a given time resolution, this does define a local timescale, but tends to zero at small times, and has no obvious characteristic value for the process as a whole. Overall, that we have such problems defining an objective timescale does not mean that no meaningful quantities with the dimensions of time can be derived from such data, but that we need to specify a model of the temporal process first (preferably deriving from a physical model of the object).

The power spectrum measures the variance (and thus the typical size of variability) at a given timescale, so we can in principle compare active galactic nuclei (AGN) with each other, or with objects like Cyg X-1. However, the absolute size of variance depends on the apparent flux of the source, so it is necessary to normalize the power spectrum to the total variance, or equivalently to normalize the light curve to the mean flux. As explained above however, we cannot be confident that we have yet made fair estimates of the variance or the mean, and thus of the normalized power spectrum. It may be that the process producing $1/f$ variations is not statistically stationary. If however we assume stationarity, our problems will be alleviated if the spectrum eventually flattens at low frequencies and steepens at high frequencies. A flattening at low frequencies is seen in Cyg X-1 and GX339-4^{10,17}. The position of such a knee

would define an unambiguous timescale, to compare with Cyg X-1 or GX339-4, but of course, as in these latter cases, such a timescale would be very long, and of no relevance to estimating black hole masses, or Doppler boosting factors. (It may tell us something about the gross geometry and/or physical conditions of the accreting regions.) We do have a limit on the knee position, $<2 \times 10^{-5}$ Hz ($n = -5$). In Cyg X-1 the knee is at $\sim 5 \times 10^{-2}$ Hz (ref. 7). If knee position scales with X-ray luminosity, the expected position is $\sim 5 \times 10^{-6}$ Hz, so we are just short of checking this hypothesis. Even more frustratingly, it seems to be normal when publishing astronomical power spectra to label the ordinate 'power in arbitrary units', for example with Cyg X-1⁷ and GX339-4¹⁴. So, even if we ignore the divergence of variance and use the sample variance to normalize the spectrum, we cannot currently tell if the power spectrum of NGC4051 is consistent with the power spectrum of Cyg X-1, with frequency scaled by the ratio of luminosities.

There have been two optical studies of AGN with which the present results can usefully be compared. First, Moore *et al.*¹⁵ have monitored the V-magnitude and polarization of BL Lac over a period of one week. The spectrum is low-frequency divergent, but a more detailed comparison is hard. No units are given for the power spectrum; the slope seems closer to $1/f^2$ than $1/f$, but the scatter is large; and the dynamic range over which there is statistical confidence is only a factor of 10. The second AGN comparison worth making is with the historical optical light curve of 3C273. Kunkel¹⁶ presents a power spectrum which again seems close to $1/f^2$ (the $1/f^2$ form justified Terrell and Olsen¹⁷ in fitting a shot-noise model). Kunkel's figure also shows an apparent low-frequency flattening, but this is probably not real. As the total sample length is 80 yr, the sample power spectrum will be a poor estimator (and probably biased) below ~ 0.05 cycles per year.

We have emphasized that to extract physical parameters from an erratic time series, it is necessary to propose a model which specifies an expected temporal form. Certainly most earlier simple interpretations of variability, and its systematics, need to be treated with great caution. From this point of view, the failure of physicists to produce models which convincingly explain $1/f$ noises in other circumstances^{10,11} is depressing. However, we can reject models (for example, simple shot noise models, models dominated by a single cooling or heating timescale), and the fundamental limits remain (for example, light travel time). Also, the fact that AGN flicker noises at various wavelengths and timescales are not all $1/f$ raises some hope that systematic differences may yield clues. Finally, we have the hope that extension to longer and/or shorter timescales will finally reveal characteristic features.

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1. Marshall, F. E., Holt, S. S., Mushotzky, R. F. M. & Becker, R. H. *Astrophys. J.* **269**, L31-L37 (1983).
2. Lawrence, A., Watson, M. G., Pounds, K. A. & Elvis, M. *Mon. Not. R. astr. Soc.* **217**, 685-699 (1985).
3. Barr, P. & Mushotzky, R. F. *Nature* **320**, 421-423 (1986).
4. Mushotzky, R. F., Holt, S. S. & Serlemitsos, P. *Astrophys. J.* **225**, L115-L118 (1978).
5. Lawrence, A. *Mon. Not. R. astr. Soc.* **192**, 83-94 (1980).
6. McHardy, I. & Czerny, B. *Nature* **325**, 696-698 (1987).
7. Nolan, P. L. *et al. Astrophys. J.* **246**, 494-501 (1981).
8. Mandelbrot, B. B. *Fractals: Form, Chance and Dimension* (Freeman, San Francisco, 1977).
9. Press, W. H. *Comments Astrophys.* **7**, 103-119 (1978).
10. Hooge, F. N., Kleinpenning, T. G. M. & Vandamme, L. K. J. *Rep. Prog. Phys.* **44**, 479-532 (1981).
11. Dutta, P. & Horn, P. M. *Rev. Mod. Phys.* **53**, 497-516 (1981).
12. Guilbert, P. W. & Fabian, A. C. *Nature* **296**, 226-228 (1982).
13. Fabian, A. C. *Paper presented at Conf. Physics of Accretion onto Compact Objects*, Tenerife, Spain, April 1986 (eds Mason, K. O., Watson, M. G. & White, N. E.) (Springer, Berlin, 1986).
14. Motch, C., Illovaitsky, S. A. & Chevalier, C. *Astr. Astrophys.* **109**, L1-L4 (1982).
15. Moore, R. L. *et al. Astrophys. J.* **260**, 415-436 (1982).
16. Kunkel, W. E. *Astr. J.* **72**, 1341-1348 (1967).
17. Terrell, J. & Olsen, K. H. *Astrophys. J.* **161**, 399-413 (1970).
18. Taylor, B. G., Andersen, R. D., Peacock, A. & Zobl, R. *Space Sci. Rev.* **30**, 479-494 (1981).
19. Turner, M. J. L., Smith, A. & Zimmerman, H. U. *Space Sci. Rev.* **30**, 513-524 (1981).
20. de Korte, *et al. Space Sci. Rev.* **30**, 495-512 (1981).

Fractal X-ray time variability and spectral invariance of the Seyfert galaxy NGC5506

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Rapid X-ray variability has long been sought after in active galactic nuclei (AGN) as an indicator of such parameters as the mass-to-energy conversion efficiency and black hole mass. Although some interesting variability information has been gained from early observations (for example see ref. 1 for a review) the faintness of AGN, combined with the short times devoted to their observation, has resulted in a very sparsely sampled and consequently partially misunderstood picture of AGN variability. The launch of EXOSAT² in May 1983 provided us with the first opportunity to study seriously AGN X-ray variability and significant variations have been detected in a number of short observations^{3,4}. Here we report the results of a long (3-day) observation of NGC5506 in which continuous rapid variation was detected. Power spectral analysis and fractal analysis both demonstrate that the variability is self-similar, that is, scale invariant, over nearly three decades of frequency, a range similar to that covered by pre-EXOSAT observations of bright galactic sources. If this behaviour is typical of all AGN then the concept of a 'two-folding' timescale is meaningless unless the fractal dimension of the general variability, as well as the specific time interval over which a specific change in flux occurs, are all noted. There is no evidence for any variation in the X-ray spectrum as a function of intensity and we show how this places strong constraints on the emission mechanism, favouring non-thermal models involving shocks.

The moderate X-ray luminosity ($L_x[2-10 \text{ keV}] = 1.4 \times 10^{43} \text{ ergs s}^{-1}$) Seyfert 2 galaxy NGC5506 was observed continuously by EXOSAT for 3 days from 24-27 January 1986 and was detected strongly in the medium energy (ME) detectors⁵ but only weakly through the thin lexan filter of the low-energy imaging telescope (LE)⁶. The highest observed signal-to-noise region of the X-ray spectrum of NGC5506 is that between 2 and 7 keV in the ME detectors, and so we present the background-subtracted light-curve in this energy range in Fig. 1. Variability on all timescales is apparent. The power spectrum of the light-curve is presented in Fig. 2. The power spectrum has a slope of ~ -1 (that is, $1/f$) in the frequency range $10^{-4.8} - 10^{-3.5}$ Hz, similar to that of the bright galactic X-ray source Cygnus X-1 (ref. 7). At higher frequencies the slope is harder to define accurately, but may steepen briefly before flattening off as it becomes dominated by Poisson noise at frequencies above $\sim 10^{-3}$ Hz. There are no obvious strong features in the power spectrum, such as the characteristic flattening in the power spectrum of Cygnus X-1 (ref. 7) at 5×10^{-2} Hz. However the fact that the power spectrum is described reasonably by a power-law does tell us that there are no preferred timescales and that variability occurs in a scale-invariant manner, at least over the range $10^{-4.8} - 10^{-3.5}$ Hz.

We now introduce another characteristic of the variability by calculating the fractal dimension of the lightcurve. (See Mandelbrot⁸ for a general introduction to fractals.) This parameterization was prompted by the similarity of the X-ray light-curve to the shape of a coastline, whose degree of irregularity is well described by a fractal dimension. A full discussion of the applicability of fractal analysis to the understanding of variability is postponed to a future paper and here we merely quote our definitions of fractal 'length' and fractal 'dimension'.

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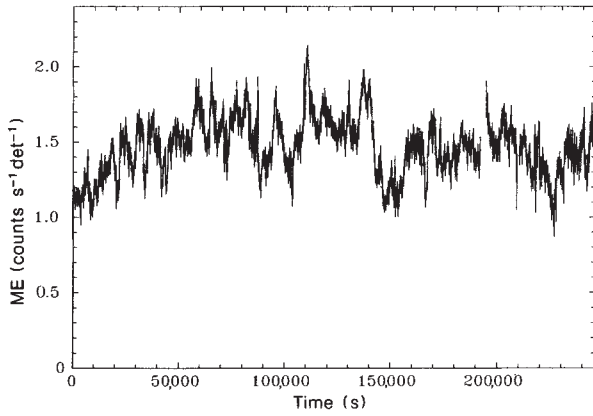


Fig. 1 X-ray light-curve of NGC5505 obtained with the EXOSAT ME in the energy range 2–7 keV. Bin size is 400 s. Error bars of $\pm 1\sigma$ are shown. $T = 0$ corresponds to 1986 January 24 21:43:32 UT.

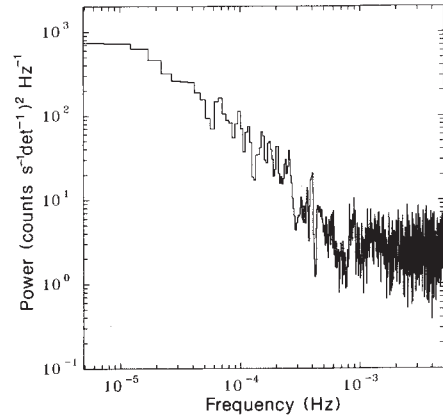


Fig. 2 Power spectrum of the light-curve shown in Fig. 1.

We define a length $L(\Delta t)$, as a function of measuring time-scale, Δt , by

$$L(\Delta t) = \frac{1}{\Delta t} \int_0^T |F(t + \Delta t) - F(t)| dt$$

and hence define a fractal dimension, D , for the light-curve by

$$D = \frac{-d \log L(\Delta t)}{d \log \Delta t}$$

Here, our dimension varies between 0 and 1 rather than between 1 and 2, as does that of a coastline, because we are considering only a (Euclidean) one-dimensional problem, that of the variation of intensity.

The measured value of $\log L(\Delta t)$ contains a contribution from the noise associated with the flux measurements. This can be calculated very simply (it has $D = 1$), given the known value of the errors. The noise is statistically independent of the signal and so can be subtracted using the relation

$$L_{\text{observed}} = (L_{\text{Signal}}^2 + L_{\text{Noise}}^2)^{1/2}$$

We performed the subtraction for three possible values of the noise associated with each measurement: (1) the average value of the error associated with each flux measurement, 0.109 counts $s^{-1} \text{ det}^{-1}$ (filled circles); (2) zero noise subtracted (open squares); and (3) an average noise value of 0.131 counts $s^{-1} \text{ det}^{-1}$ (stars). This latter value is chosen somewhat arbitrarily but corresponds to the largest value that can be subtracted without making $L_S(100 \text{ s}) < 0$. The resultant plot of $\log L_S(\Delta t)$ against $\log(\Delta t)$, after subtraction of the noise, is shown in Fig. 3.

$\log L_S$ appears to be linearly related to $\log \Delta t$ down to $\Delta t = 100 \text{ s}$, although for $\Delta t \leq 400 \text{ s}$ the envelope provided by the allowed error in our determination of the noise cautions against placing too much confidence in the relationship. However for $\Delta t > 400 \text{ s}$ the relationship is well constrained and remains so up to the limits of observation (10^5 s).

We have shown that the X-ray variability of NGC5506 is well described by a fractal dimension (0.6) over nearly 3 decades in frequency and therefore that the variability is self-similar, and hence scale-invariant, over this range. The fractal dimension we measure is close to that of (Euclidean one-dimensional) turbulence (0.5) and so similar random processes, such as randomly separated outbursts of random amplitude, are probably responsible for the irregular shape of the X-ray light-curve. The same result is broadly understandable from consideration of the large amplitudes visible in the light-curve; for these to be produced by random superposition of many of the smaller amplitudes we see is statistically extremely unlikely.

The fractal dimension cannot remain greater than 0.5 down to infinitesimally small timescales as this would imply infinite energy. However the present data tell us that any decrease in the dimension, that is, anything which we might associate with a minimum timescale, must occur at timescales shorter than 400 s. Thus, until we can measure deviations from self-similarity, we can only place a lower limit on the rate of change of luminosity with time. The limit in this case ($\sim 10^{39.5} \text{ ergs s}^{-2}$) only implies an efficiency of mass-to-energy conversion of 0.5% and hence places no strong constraints on black hole models.

It can be shown (D. Raine, personal communication) that, within certain limits, if a power spectrum has a power-law slope, P , the corresponding fractal plot will have a dimension, D , where

$$P = 2(D - 1).$$

Thus, the fractal dimension we measure here agrees, within the uncertainties, with the value $P = -1$, that is, $1/f$ noise, which we directly measure from the power spectrum (Fig. 2) over the range $10^{4.8} - 10^{3.5} \text{ s}$.

The scale-invariant temporal variability of NGC5506 makes it impossible to identify any particular timescale as, for example, a dynamical or thermal timescale. If this is a problem common to active galaxies in general it will not be possible to calculate such parameters as a black hole mass from the characteristics of the variability. Another frequently discussed parameter, the two-folding timescale, also becomes meaningless for self-similar light-curves, as can easily be seen from fractal analysis. For $D \neq 0$ the local derivative is not determined and the value which we might try to define as the derivative, $\Delta F / \Delta t$, depends on Δt with a mean value of

$$\left| \frac{\Delta F}{\Delta t} \right| \propto \Delta t^{-D}$$

independent of the actual value of F . However for self-similar variability F can, in principle, take any value. Thus the mean value of F we measure during any observation will depend on when the observation occurred and so any timescale defined by $F / (dF/dt)$, as is conventionally the case, will also depend on the local mean value of F . Only if we restrict ourselves to considering changes within a well-observed time interval, or if self-similarity breaks down at large times and the mean value of F is approximately constant, can we define a universal two-folding time for a source. In this case we still need to measure a change by a factor of 2 to define such a timescale. If the observed change was smaller or larger than a factor of 2, we would have to determine the dimension of the variability and apply a correction factor reflecting the dependence of $|\Delta F / \Delta t|$ on Δt . For example a two-folding timescale, Δt_2 , can be calculated from observed variations in flux of 10% in a time Δt_1 by

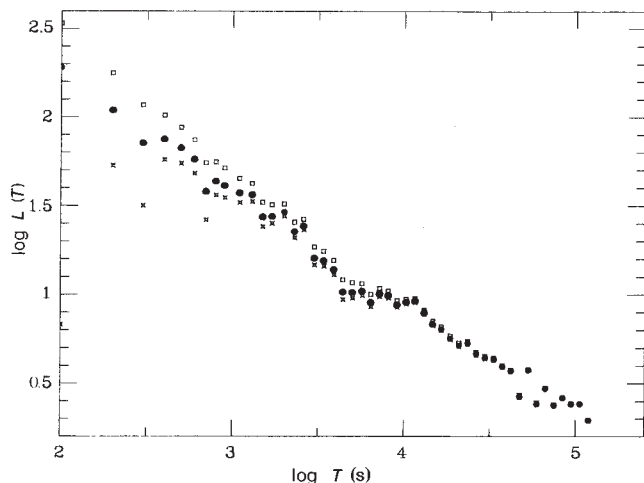


Fig. 3 Shows log of total 'length' plotted against 'timescale' (as described in the text) for the light-curve shown in Fig. 1. The slope of the curve is $-D$, where D is the fractal dimension.

$\Delta t_2 = \Delta t_1 / (0.1)^{1/(1-D)}$. The relationship between two-folding time and luminosity shown by Barr and Muchotzky⁹ needs to be redrawn with this correction applied.

The source is heavily cutoff ($N_H = 2.8 \times 10^{22} \text{ cm}^{-2}$) and the average LE count rate ($1.9 \times 10^{-3} \text{ counts s}^{-1}$ [0.05–1.0 keV]) is in very good agreement with a simple extrapolation of the ME spectrum to low energies. The LE count rate is too low for us to study low-energy variability; however, the ME count rate is high enough to allow us to investigate the variation of the medium-energy spectrum as a function of intensity. We have therefore divided the observation into twelve equally separated intensity levels covering the full range of variability and have fitted simple power-law plus absorbing column models to the data in each level. The resultant spectra revealed no evidence for change in either the absorbing column or energy index. If we assume that the absorbing column does not change, which seems reasonable as the timescale for large-intensity variations is very short ($\sim$ hours), we can fix the absorbing column at its best-fit value [$2.8 \times 10^{22} \text{ cm}^{-2}$] and so derive improved estimates of the variation in power-law index. In Fig. 4 we plot the variation of index thus derived (here, for computational ease, we show only those data derived from half 2 of the ME). There is no evidence at all for any variation. The best-fit value is 0.84 ± 0.04 . The spectral parameters we derive are in reasonable agreement with previous determinations^{10,11} and indicate that there are no long-term spectral variations in this source.

We have constructed a hardness ratio (not shown) between the counts in the energy range 4.5–7 keV and 2–4.5 keV. The ratio is approximately constant throughout the observation and certainly does not follow the rapid rises and falls apparent in Fig. 1. Therefore the constancy of the spectral index shown in Fig. 4 is not a result of a superposition of, say, a harder spectrum during outbursts and a softer spectrum during declines; rather, the spectrum must be approximately constant at all times.

By means of fractal and power spectral analysis we have shown that the X-ray variability of NGC5506 is self-similar, or scale invariant, over nearly 3 decades of frequency with a fractal dimension close to that of random processes such as turbulence. Such random activity could be accounted for in many ways; for example by the release of stored energy in magnetic fields or perhaps pairs, by turbulent accretion and associated Doppler enhancements or by shocks. Any such activity must not, however, produce any variation in the observed X-ray spectrum and so 'constant seed' (that is, variable temperature) thermal Compton models¹² can be rejected.

Simultaneous optical photometry was obtained for part of the X-ray observation by Ricker and Vanderseck. These data

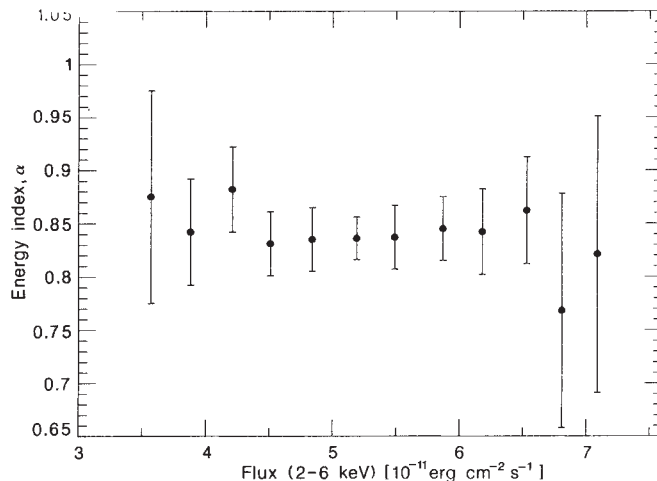


Fig. 4 Power-law spectral index as a function of intensity.

are not yet fully reduced but initial indications are that no variability amplitudes as large as those shown in Fig. 1 were detected. If full analysis confirms this, 'constant temperature' (and hence variable seed) thermal Compton models may also be in some difficulty (see NGC4051 where Lawrence *et al.*³ indicate that seeds may come from the optical region).

The release of stored magnetic field energy into the thermal energy of a plasma, such as occurs in RS CVn systems¹³, can also probably be rejected as this leads to a rise in temperature during the outburst and a fall during the decline. The fact that the X-ray spectrum fits a power-law very well (but is not well-fitted by a simple thermal bremsstrahlung model) also argues against release of stored magnetic energy, as does the fact that the exponential decay of such bursts would produce a power spectrum which is not a simple $1/f$ power-law².

If the source is a regime where pairs are important, a slight change in the gamma-ray luminosity, such as may accompany a variation in accretion rate, can be reflected in a large variation in the X-ray luminosity with little or no change of spectral slope¹⁴.

Finally we note that perhaps the simplest method of producing spectrally invariant intensity variations is with shocks. If the underlying electron distribution has a power-law form then simple compression and expansion of the radiating regions will alter the intensity (of synchrotron or synchrotron self-Compton emission) without change of slope. Even if the initial electron distribution is not a power-law, then shocks are likely to turn it into one eventually.

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- McHardy, I. M. *Space Sci. Rev.* **40**, 559–584 (1985).
- Taylor, B. G., Andresen, R. D., Peacock, A. & Zobl, R. *Space Sci. Rev.* **30**, 479–494 (1981).
- Lawrence, A., Watson, M. G., Pounds, K. A. & Elvis, M. S. *Mon. Not. R. astr. Soc.* **217**, 685–699 (1985).
- Pounds, K. A., Turner, T. J. & Warwick, R. S. *Mon. Not. R. astr. Soc.* **221**, 7–10 (1986).
- Turner, M. J. L., Smith, A. & Zimmerman, H. U. *Space Sci. Rev.* **30**, 495–512 (1981).
- de Korte, P. A. J. *et al. Space Sci. Rev.* **30**, 495–512 (1981).
- Nolan, P. L. *et al. Astrophys. J.* **246**, 494–501 (1981).
- Mandelbrot, B. B. *Fractals: Form, Chance and Dimension* (Freeman, San Francisco, 1977).
- Barr, P. & Mushotzky, R. F. *Nature* **320**, 421–423 (1986).
- Maccacaro, T., Perola, G. C. & Elvis, M. S. *Astrophys. J.* **257**, 47 (1982).
- Reichert, G., Muschotzky, R. F., Petre, R. & Holt, S. *Astrophys. J.* **296**, 69 (1985).
- Guilbert, P. W., Fabian, A. C. & Ross, R. R. *Mon. Not. R. astr. Soc.* **199**, 763–774 (1982).
- Pye, J. P. & McHardy, I. M. *Mon. Not. R. astr. Soc.* **205**, 875–888 (1983).
- Fabian, A. C., Blandford, R. D., Guilbert, P. W., Phinney, E. S. & Cuellar, L. *Mon. Not. R. astr. Soc.* **221**, 931 (1986).

Scintillation of a radio source observed through the tail of comet Halley

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On 29 March 1986 between 16:00 and 23:00 UT we observed the strong compact radio source 1827–360 as the plasma tail of comet Halley swept across the source. We noted the appearance of weak intensity fluctuations during the interval 17:00 to 19:00 UT when the Sun, the comet's nucleus and the radio source were accurately aligned. We interpret this behaviour as the result of diffraction of radio waves by electron-density irregularities in the plasma tail. At a distance 5.4×10^6 km downstream from the cometary nucleus the central part of the plasma tail contained electron density irregularities about 14 times as strong as those in the undisturbed solar wind.

The source 1827–360 is known to exhibit interplanetary scintillation¹ and has the turnover in the low-frequency radio spectrum² often shown by very compact radio sources. The high scintillation flux¹ of 10.6 Jy out of a total 408 MHz flux density³ of 25.8 Jy makes it an ideal source for detecting scintillation from the plasma tail of Comet Halley. The observations were made with a dual-channel 408 MHz receiver installed on the Parkes 64-m telescope. The system temperature of each channel was ~ 120 K and bandwidth 5 MHz. The operation of the telescope and data taking were computer-controlled; the total power in each polarization channel was sampled at 50 Hz in blocks of 40.96 s and the readings stored for subsequent analysis. Active low-pass filters with a cut-off at 23 Hz minimized aliasing of frequencies above the Nyquist frequency of 25 Hz. Off-line analysis consisted of: (1) subtracting a quadratic baseline from each block of data and rejecting interference spikes; (2) plotting the modified data and computing its r.m.s. variation; (3) computing a power spectrum with frequency resolution of ~ 0.05 Hz; (4) averaging the two polarization channels and averaging several data blocks.

The path of the comet projected on to the plane of the sky is shown in Fig. 1. The nucleus of Halley had a geocentric velocity of 39.94 km s^{-1} and its elongation from the Sun was 89° . The topocentric and heliocentric distances were 0.576 and 1.143 AU respectively; the projected distance from the nucleus at which the tail passed in front of the source was 5.4×10^6 km.

Figure 2a shows a block of data taken at 17:35 UT with the telescope pointing 1° away from 1827–360; Fig. 2b shows a block of data taken at 18:10 UT while pointing at the source. The noticeably increased noise fluctuation level in Fig. 2b yields a scintillation index (defined as $m = \text{r.m.s. variation in flux/scintillating flux density}$) of $m = 0.064$. Figure 3 is a plot of the scintillation indices measured over the four observing days. The scintillation index has been computed by subtracting the variance off source from that on source, with a correction to account for the 12.5% increase in the system temperature when on source. Each scintillation index is an average over 15 min, the larger gaps on 29 March being due to off-source checks.

The scintillation index on 29 March was significantly higher for much of the 7.5-h observing interval than it was on the preceding and following days. In particular, during the interval 17:00–19:00, when Fig. 1 shows that the centre of Halley's plasma tail was passing in front of the source, the scintillation index reached 0.062, which is about four times higher than the average of 0.016 for the other three days. Other features of Fig. 3 are the very low scintillation indices between 19:30 and 21:00 UT

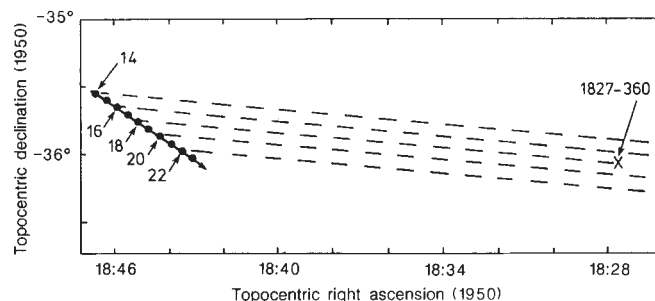


Fig. 1 Equiangular projection on to the plane of the sky of the path of Comet Halley with respect to the radio source 1827–360 (cross). The position of the nucleus is shown at hourly intervals (UT) on 29 March 1986. The dashed lines represent the extended Sun-Halley vectors at selected times.

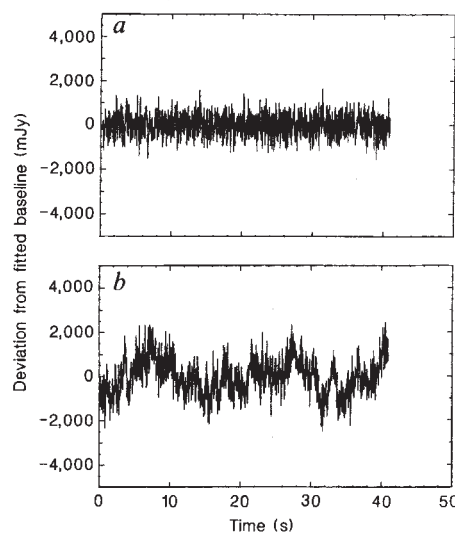


Fig. 2 Samples of data taken during the occultation. Panel *a* refers to a block of data recorded off source at 17:35 UT on 29 March 1986; *b* shows a block of data recorded on source at 18:10 UT.

and a secondary maximum of ~ 0.040 between 22:00 and 22:45 UT.

We have examined the possibility that ionospheric scintillation may have been responsible for the peaks in Fig. 3 by checking the F2 critical frequencies and frequency spreading determined by a sounder operated by the Australian Ionospheric Prediction Service at Canberra, ~ 300 km SSW of Parkes; the radiation from 1827–360 would have been passing through the F2 layer above Canberra near the time of the maximum in scintillation index from 17:00 to 19:00 UT. The 15-min ionograms showed a reasonable degree of spread- f throughout our observing interval, but the spreading was most severe between 18:30 and 19:45 UT when, in fact, the measured scintillation index was dropping rapidly. The geomagnetic K_p indices of 3, 2+ over the observing interval on March 29 were close to those on neighbouring days. These considerations, together with the fact that the peak in the scintillating power spectrum (see Fig. 4) occurs at ~ 0.1 Hz (much too high for normal ionospheric scintillation), effectively eliminates an ionospheric origin for our scintillations.

We do not believe that our results can be explained by the chance occurrence of an enhanced patch of solar wind turbulence, because: (i) the plasma density and velocity in the solar wind as measured by the spacecraft Sagigake⁴ were particularly stable, with values characteristic of the undisturbed plasma for many days in late March and early April 1986; (ii) comparison observations of 1827–360 on three days (see Fig. 2) gave very low values of scintillation index; (iii) the scintillations were strongest when the extended Sun-comet vector was passing over the source.

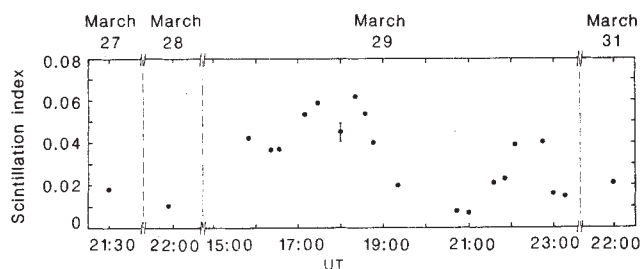


Fig. 3 Scintillation indices computed from data taken over the four days on which 1827–360 was observed. Each point represents an average over ~15 min. A typical standard error bar is shown. Off-source measurements were made during the longer gaps on 29 March.

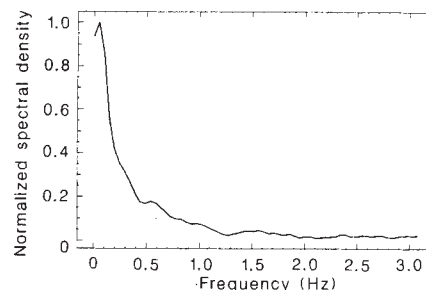


Fig. 4 A scintillation power spectrum computed from ~1 h of on-source data near the peak of scintillation index in Fig. 3. The frequency resolution before Hanning was 0.0488 Hz. Spectral estimates have been normalized to the maximum value.

Figure 4 shows the scintillation power spectrum obtained by averaging over ~1 h of data near the peak of scintillation index in Fig. 2. The spectrum was computed by subtracting the average of 60 off-source spectra from the average of 80 on-source spectra. The spectral resolution before Hanning is 0.0488 Hz and the spectral densities have been normalized to the maximum value. It is clear that most of the variance is contained in frequencies below 2 Hz. This spectral behaviour is similar to that of ordinary interplanetary scintillation in the weak scattering regime⁵ except that more of the variance is at lower frequencies. We have made computations of scale size and r.m.s. electron density fluctuation in the comet tail on the very reasonable assumption that we are dealing with weak scattering in a thin diffracting screen.

We have no photograph of Halley's plasma tail on the night of our observation (near full moon), but a UK Schmidt plate taken by D. Malin (personal communication) on 12 March gives a good impression of the structure that was probably present. At the distance of 5.4×10^6 km from the nucleus relevant to our present analysis the bright central part of the plasma tail had a projected width of 4.8×10^5 km and would have drifted across the radio source in ~3 h. From the scattering theory developed by Salpeter⁶ the correlation length or scale size is given by $a = v/2\pi f_2$, where v is the velocity of the diffraction pattern across the observer and f_2 is the width of the scintillation power spectrum at the exp(-0.5) point. It is unlikely at a distance 5.4×10^6 km (0.036 AU) downstream from the nucleus that the cometary plasma would have been accelerated to the solar wind velocity. Observations of plasma condensations in the tail of comet Kohoutek by Jockers⁷ yielded velocities < 100 km s⁻¹. Here we assume that the total translational velocity of the plasma with respect to the line of sight to 1827–360 was $v = 90$ km s⁻¹, which, when combined with $f_2 = 0.14$ Hz measured from the power spectrum of Fig. 4, gives $a \approx 100$ km. This scale size is typical of ordinary interplanetary scintillations when radio sources are observed in the weak scattering regime⁵. For a gaussian electron density correlation function the r.m.s. phase deviation across the wavefront emerging from the screen⁵ is

$$\phi_0 = (2\pi)^{1/4} r_e \lambda (aL)^{1/2} \Delta N,$$

where $r_e = 2.82 \times 10^{-13}$ cm is the classical electron radius, L is the thickness of the screen and ΔN is the r.m.s. electron density deviation. For weak scattering the scintillation index⁶ is $m = \sqrt{2}\phi_0$, from which in our case ($\bar{m} = 0.062$) $\phi_0 = 0.044$ rad. Then making the reasonable assumption that the screen thickness is approximately equal to the width seen on the photograph of 12 March ($L \approx 4.8 \times 10^5$ km) we deduce that $\Delta N \approx 1.9$ cm⁻³. This value is an order of magnitude higher than the value of ΔN ordinarily found in the solar wind at heliocentric distances of ~1 AU (see, for example, Fig. 9 of Cohen *et al.*⁵). We have also estimated ΔN by using the more realistic Kolmogorov power-law spectrum of irregularity sizes. Equations (16) and (17) given by Lee⁸ with an inner scale of 100 km (the scale size from the gaussian analysis) and an outer scale of 4.8×10^5 km (the width of the plasma tail) were solved to give $\Delta N = 1.8$ cm⁻³, which is unexpectedly close to the value from the gaussian spectrum.

Scintillation experiments do not provide a direct measurement of the average electron density in a comet tail. If the r.m.s. electron density deviation which is responsible for the scintillations is proportional to the average electron density, then at 5.4×10^6 km downstream from the nucleus on 29 March the average electron density was ~ 70 cm⁻³ (14 times the solar wind value of ~ 5 cm⁻³ measured⁹ under similar undisturbed conditions upstream from Halley during Giotto's approach on 14 March 1986).

It is instructive to compare our results with those of Ananthakrishnan *et al.*¹⁰ from comet Kohoutek and Alurkar *et al.*¹¹ from the recent apparition of comet Halley. Lee⁸ has shown that the 327 MHz scintillations observed during the occultation of PKS2025–15 by the tail of Kohoutek at a distance of $\sim 5.5 \times 10^5$ km (0.0037 AU) from the nucleus could be explained by a Gaussian spectrum of turbulence having a scale of $a \approx 800$ km and $\Delta N \approx 22$ cm⁻³. These values should be compared with those from our experiment of $a \approx 100$ km and $\Delta N \approx 1.9$ cm⁻³ at a distance of 5.4×10^6 km (0.036 AU) from Halley's nucleus.

A better comparison is with the 103 MHz scintillations¹¹ of PKS2314+03 during occultation by Halley's tail in December 1985. Postulating a gaussian irregularity spectrum with $a \approx 100$ km, these authors claim a rapid variation in ΔN with radial distance along the tail from $\Delta N = 10$ cm⁻³ at 0.12 AU to $\Delta N = 3$ cm⁻³ at 0.18 AU. In subsequent correspondence S. K. Alurkar *et al.* (personal communication) have agreed that their assumed tail width of 10⁵ km is probably underestimated by a factor of approximately five, so that ΔN should be reduced by $\sim \sqrt{5}$. The evidence that this is a real radial variation in ΔN is not very convincing—the observations occupied only ~10 min each day and we know from Jockers⁷ observations of comet Kohoutek that plasma condensations move out along the tail with sufficient velocity to change their radial distribution from day to day. We prefer to average the 103 MHz results to give $\Delta N \approx 2.8$ cm⁻³ at 0.15 AU from the nucleus. Therefore, assuming that rate of ionization of nuclear material had not changed significantly from December 1985 to March 1986, there is little, if any, convincing radial variation in ΔN .

The results from comet Kohoutek^{8,10} are not compatible with the Halley measurements. The 103 and 408 MHz measurements of Halley indicate that if there is a radial gradient in ΔN , it is in the direction of the turbulence level decreasing slowly with approach to the nucleus. On the other hand, the r.m.s. electron density for comet Kohoutek at 0.0037 AU from the nucleus is an order of magnitude higher than an extrapolation of our results inward would suggest.

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1. Milne, R. G. thesis, Univ. Sydney (1976).
2. Slee, O. B., Siegman, B. C. & Mulhall, P. S. *Proc. Astron. Soc. Aust.* **4**, 278–704 (1982).
3. Large, M. L., Mills, B. Y., Little, A. G., Crawford, D. F. & Sutton, J. M. *Mon. Not. R. astr. Soc.* **194**, 693–704 (1981).

4. Oyama, K. I., Hirao, K., Hirano, T., Yumoto, K. & Saito, T. *Nature* **321**, 310–313 (1986).
5. Cohen, M. H., Gundermann, E. J., Hardebeck, H. E. & Sharp, L. E. *Astrophys. J.* **147**, 449–466 (1967).
6. Salpeter, E. E. *Astrophys. J.* **147**, 433–448 (1967).
7. Jockers, K. *Icarus* **47**, 397–411 (1981).
8. Lee, L. C. *Astrophys. J.* **210**, 254–257 (1976).
9. Reme, H. *et al.* *Nature* **321**, 349–352 (1986).
10. Ananthakrishnan, S., Bhandari, S. M. & Rao, A. P. *Astrophys. Space Sci.* **37**, 275–282 (1975).
11. Alurkar, S. K., Bhonsle, R. V. & Sharma, A. K. *Nature* **322**, 439–441 (1986).

Protracted climatic effects of massive smoke injection into the atmosphere

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Climate perturbations caused by sudden injections of aerosols into the atmosphere have received increased attention with the realization that fires started by a nuclear war might release sufficient quantities of smoke to block sunlight from the Earth's surface and dramatically reduce temperatures over land^{1–3}. Three-dimensional atmospheric general circulation models which include aerosol transport indicate that much of the smoke could survive in the atmosphere for months or more^{4–6}, implying significant climatic effects on such timescales, but these models generally assume fixed sea surface temperature and sea ice, and therefore cannot properly simulate cooling for more than a few weeks. This report describes a general circulation model run in which the foregoing assumptions are relaxed and the climate impact of massive smoke loading is assessed for four months of simulated time. Two competing long-term effects are apparent: positive feedback on surface cooling due to enhanced sea ice formation, and surface warming at some latitudes due in part to heat transport from the upper troposphere, where smoke aerosols absorb sunlight.

It is instructive to compare results from the general circulation model (GCM) to those obtained by Robock⁷ using a one-dimensional (in latitude) energy balance model (EBM) in multi-year simulations of nuclear war climate effects. In the present study, total column absorption optical depth of smoke τ is prescribed as a function of time to be twice the value normally assumed by Robock except that we restrict $\tau \leq 3$, that is, underneath the smoke $\tau = 3$ for the first 30 days and thereafter τ decreases with a half life of 30 days. This prescription ensures that GCM results of interest are obviously significant compared to model-generated variability (that is, weather fluctuations) and also that during the first 20 days after aerosol injection the smoke concentrations assumed for this study are identical to those used in our earlier GCM experiments with prescribed sea surface temperature (SST)^{8,9}. In all studies discussed here, including Robock's, smoke is assumed to exist in a uniform band of latitudes between 30° and 70° N and is injected into the atmosphere at approximately the Northern Hemisphere spring equinox. In the GCM runs smoke is assumed to be distributed between 1 and 10 km altitude at constant mixing ratio.

The assumed initial condition $\tau = 3$ in the Northern Hemisphere mid-latitudes is equivalent to 34×10^{12} g of amorphous elemental carbon with absorption cross section $10 \text{ m}^2 \text{ g}^{-1}$ and corresponds to 'baseline' estimates of nuclear war smoke production published by the US National Academy of Sciences² and the International Council of Scientific Unions³. However, longer term optical depths, $\tau = 2.4$ at 40 days and 0.5 at 110 days, greatly exceed values implied by GCMs which compute smoke removal^{5–6,10}, and of course the assumption that smoke is fixed in space is unrealistic. Thus, the present study is an intercomparison of climate model responses to hypothetical perturbations, with application to nuclear war climate effects; it is not an attempt to directly simulate 'nuclear winter'. The

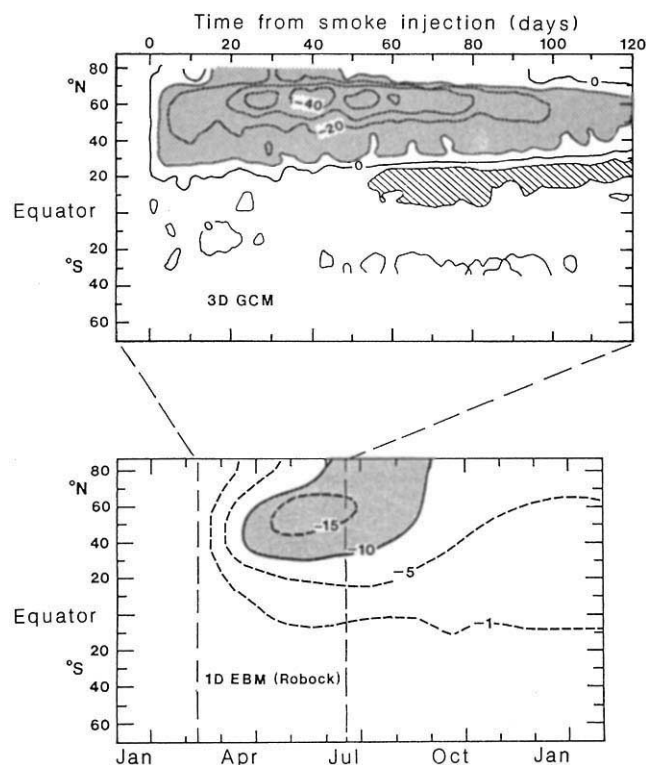


Fig. 1 Zonally averaged land surface temperature difference in °C between prescribed smoke experiments and controls. The upper panel shows results obtained in the present study; the lower panel is redrawn from ref. 7 and shows results from a one-dimensional energy balance climate model. In both cases solid line contours are drawn at 10°C intervals, and shaded areas indicate temperatures more than 10°C cooler than control runs while hatched areas indicate temperatures more than 10°C warmer than control runs. GCM results are shown for 130 days and the thin dashed lines indicate the corresponding time period in Robock's simulation. Results from the GCM are not plotted at latitudes which are more than 90% ocean.

use of extreme smoke amounts and the assumption that smoke does not spread beyond the Northern Hemisphere mid-latitudes mean that long-term temperature effects are probably overestimated by the present study. Indeed, in reporting a long-term 'nuclear winter' simulation including smoke spreading by a two-level GCM, Stenchikov¹¹ does not elucidate the surface temperature feedback processes discussed below, perhaps because these processes are less obvious with more realistic (smaller) smoke concentrations.

The GCM used in this study is the NCAR (National Center for Atmospheric Research) Community Climate Model (CCM)¹². Results presented here were obtained with a research version of the CCM developed by Washington and Meehl^{13,14} for studies of the CO₂ greenhouse effect. In this model SSTs are computed assuming a static mixed layer of constant heat capacity, and sea ice is computed with a simple thermodynamic model. This GCM, hereafter referred to as the CCM/mixed layer model, was modified as described by Covey *et al.*^{8,9} to include absorption of sunlight by smoke. Similar representations of mixed layer heat capacity and sea ice are used in Robock's EBM. (The earlier studies of Covey *et al.*^{8,9} made use of a version of the CCM in which SST and sea ice are prescribed at seasonally varying climatological values¹⁵; this model will be referred to as the prescribed SST version.)

Figure 1 shows the smoke-induced land surface cooling (that is, the temperature difference in °C between prescribed smoke experiments and controls) averaged over longitude and plotted as a function of latitude and time. The upper panel shows results

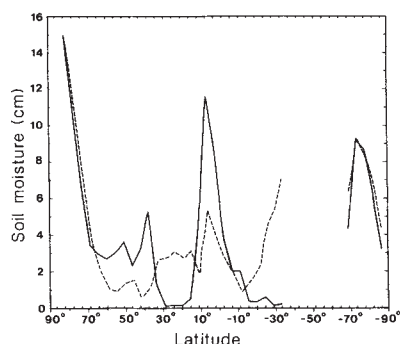


Fig. 2 Soil moisture content averaged over longitude and over days 110 to 120 after smoke injection (that is, the final 10 days of the GCM simulation) for the smoke-perturbed case (solid line) and the control case (dashed line). Results are not plotted at latitudes which are more than 90% ocean.

from the CCM/mixed layer model and the lower panel shows results from Robock's EBM ('Run 4' of ref. 7). During the first half of the 120 day simulation with the CCM/mixed layer model, land surface cooling underneath the assumed smoke cloud is about twice that exhibited by Robock's model. Maximum cooling in the GCM is 47 °C at day 37 and 60° N latitude (poleward of the sea ice margin, slightly to the north of maximum cooling in Robock's model). During this time period smoke optical depths assumed in both the CCM/mixed layer model and Robock's simulation are > 1 , so the discrepancy in the amount of cooling between the two models is difficult to explain by the difference in smoke loading. The CCM/mixed layer model may exaggerate the amount of cooling because it overpredicts the unperturbed amount of sea ice, possibly due to neglect of poleward heat transport by the oceans^{13,14}; the maximum temperature drop exhibited by the CCM/mixed layer model is almost twice that of the prescribed SST version during the time that smoke loadings in the two GCMs are identical. On the other hand, Robock's model may well underpredict the maximum degree of land surface cooling because its limited resolution—10° latitude, no longitude resolutions within land and ocean sectors, no height resolution—requires the cooling to be spread out over a large volume of space. The latter consideration may also explain in part the rapid cooling exhibited by the CCM during the first few days after aerosol injection⁸; the EBM, in contrast, takes several weeks to obtain land surface cooling > 10 °C. Another factor contributing to rapid initial cooling in the GCM is atmospheric dynamics, not included in the EBM. An examination of the lower tropospheric wind and temperature fields (not shown) indicates that ocean-to-

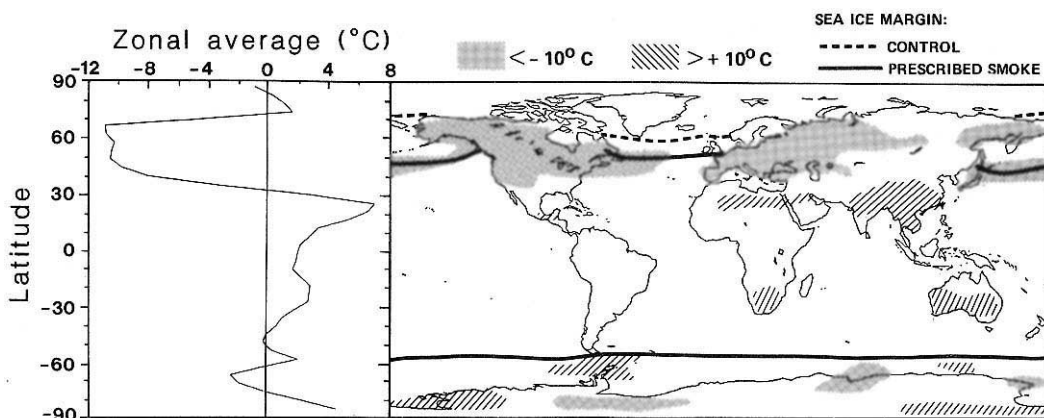
land temperature advection takes about two weeks to increase to its maximum value as the circulation in the perturbed case evolves to a more zonally symmetric state. Two weeks is approximately the thermal relaxation time of the lower troposphere, that is, the quantity $C_p T g^{-1} \Delta P / (\sigma T^4)$ where C_p is the heat capacity of air at constant pressure, T is the air temperature, g is the acceleration of gravity, ΔP is the pressure difference between the surface and the 85 kPa pressure level, and σ is the Stefan-Boltzmann constant.

Unlike the situation early on, from about 100 to 120 days after smoke injection the amount of cooling in the CCM/mixed layer model is about as small or smaller than that in Robock's EBM, despite doubled smoke loading. Furthermore, throughout the second half of the CCM simulation land surface temperatures immediately south of the assumed smoke cloud are significantly warmer than control values (see the hatched areas in Fig. 1). The EBM's surface temperatures are everywhere colder when smoke is injected into the model, for the following reason. Since the EBM has no height resolution the method of assuming smoke is to simply decrease the solar constant by the amount corresponding to absorption by the appropriate optical depth. This situation is quite different from that expected in the real atmosphere, in which smoke warmed by absorption of sunlight should overlie a cool surface (see Fig. 1a in ref. 8). It is possible that in the CCM simulation some of the heat from the upper troposphere is eventually transported (presumably via infrared radiation¹⁶) down to the surface, reversing long-term surface cooling in those areas which are not covered by smoke. This effect would no doubt be smaller in a more realistic treatment of protracted nuclear war-generated smoke, which would be more widespread, thinner, less black, and higher than the idealized fixed smoke considered here; nevertheless it is possible that the effect could moderate long-term surface cooling.

Partial support for the foregoing hypothesis comes from an analysis of the GCM's land surface energy budget averaged over the final 10 days of the model run. At 20° N latitude infrared flux from the atmosphere to land surface is 92 W m⁻² greater in the smoke-perturbed case than in the control case. A smaller warming effect (28 W m⁻²) is produced by loss of upward latent heat flux due to a drying out of soil moisture in the 15–30° N latitude zone (Fig. 2). The latter type of warming mechanism has been observed in a previous GCM simulation of nuclear war-generated smoke effects¹⁷. However, underneath the smoke (47° N) the dominant change in surface energy balance is between decreased absorption of solar radiation and decreased upward infrared and sensible heat fluxes; within this latitude band, the infrared flux received by the surface from the atmosphere is not significantly greater in the smoke-perturbed case than in the control case.

The differences in soil moisture between the control and

Fig. 3 Surface temperature difference (prescribed smoke case minus control case) averaged over days 110 to 120 after smoke injection. On the left side the zonal average is shown as a function of latitude. On the right side the geographical distribution is shown, with shaded areas indicating greater than 10 °C cooling and hatched areas indicating greater than 10 °C warming; also the control case (thick dashed line) and perturbed case (thick solid line) sea ice margins are indicated (the two coincide in the Southern Hemisphere).



perturbed cases (Fig. 2) are for the most part as expected from previous experiments with the CCM⁹. In general, the CCM responds to fixed upper tropospheric smoke loadings by decreasing cloudiness where the smoke is heated by sunlight while increasing cloudiness at lower levels, resulting in only small changes in precipitation underneath the smoke. (This result is not consistent with results from the Oregon State University two-layer model¹⁷ and may be due to the CCM's assumptions of 100% relative humidity immediately above the surface together with boundary layer condensation at only 80% relative humidity.) Accordingly, soil moisture is greater underneath the smoke in the perturbed case, where surface cooling inhibits evaporation. As pointed out above, soil moisture essentially disappears from latitudes 15–30° N, where surface warming from enhanced downward infrared radiation increases evaporation during the first half of the smoke-perturbed model integration. For latitudes 15–30° S, the expected location of the downward branch of an enhanced cross-equatorial Hadley circulation in the perturbed case (see Fig. 4b in ref. 8), a decrease in precipitation leads to the drying out of soil moisture apparent in Fig. 2. Finally, soil moisture more than doubles in the perturbed case in a narrow latitude band centered on 8° N due to a burst of precipitation during the last 10 days of the model integration, which occurs for unknown reasons.

The geographical distribution of surface cooling and sea ice averaged over the final 10 days of the CCM/mixed layer model simulation is depicted in Fig. 3. As expected from Robock's EBM results⁷ the extent of sea ice is significantly greater in the smoke-perturbed case. The GCM obtains greatest cooling in the western parts of the North American and Eurasian land masses, downwind of the sea ice, in contrast to early times when maximum cooling occurs in the eastern parts⁸. Cooling of Europe due to enhanced North Atlantic sea ice has a palaeoclimatic analogue: the Younger Dryas period, 10,000–11,000 years ago, exhibited this phenomenon according to both observational¹⁸ and model¹⁹ studies.

In summary, the three-dimensional GCM simulation, while qualitatively confirming a positive feedback due to sea ice that enhances long-term cooling due to nuclear war-generated smoke, reveals additional effects that can moderate or perhaps even reverse long-term cooling. The latter appear to dominate by the end of the GCM run, when the extent of land area that is cooled in excess of 10° C is much smaller than it is in the analogous EBM experiment ('Run 4' in Fig. 1 of ref. 7) despite the fact that the GCM experiment assumed more smoke. However, both classes of effects are probably exaggerated by the assumption of very large quantities of smoke that is fixed in space, and by other model simplifications such as lack of ocean heat transport (leading to overprediction of sea ice). It is therefore necessary to perform more realistic GCM experiments, particularly including model-computed smoke transport and removal, in order to quantitatively assess the relative importance of the positive and negative feedbacks discussed here.

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10. Thompson, S. L., & Giorgi, F. *J. geophys. Res.* (in the press).
11. Stenchikov, G. L. in *The Night After ... Climatic and Biological Consequences of Nuclear War* (ed. Velikov, Y.) 53–82 (Mir, Moscow, 1985).
12. Pitcher, E. J. *et al. J. atmos. Sci.* **40**, 580–604 (1983).
13. Washington, W. M. & Meehl, G. A. *J. geophys. Res.* **89**, 9475–9503 (1984).
14. Meehl, G. A. & Washington, W. M. *J. phys. Oceanogr.* **15**, 92–104 (1985).
15. Chervin, R. M. *J. atmos. Sci.* **43**, 233–251 (1986).
16. Cess, R. D., Potter, G. L., Ghan, S. J., & Gates, W. L. *J. geophys. Res.* **90**, 12937–12950 (1985).
17. Ghan, S. J., MacCracken, M. C. & Walton, J. J. *Lawrence Livermore natn. Laboratory tech. Not.* UCRL-92324 (1985).
18. Broecker, W. S., Peteet, D. M. & Rind, D. *Nature* **315**, 21–26 (1985).
19. Schneider, S. H., Peteet, D. M., & North, G. R. in *Abrupt Climate Changes* (eds Berger, W. H. & Labeyrie, L.) (Reidel, Dordrecht, in the press).

Trapped radicals in titania gels

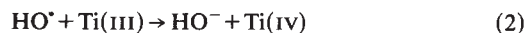
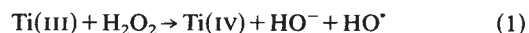
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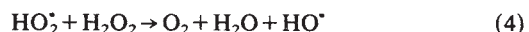
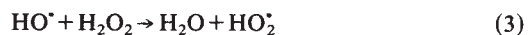
Free radicals are in general very reactive entities capable only of transient existence. Nevertheless, several methods have been devised for their detection and identification, including stabilization by matrix isolation procedures¹ and detection by spectroscopic methods. Radicals can be readily produced by oxidation–reduction processes where electrons are transferred from the solute to the matrix or conversely. Here we report the first production of trapped O₂^{•−} free radicals generated in a titania-gel matrix by the initial reaction of Ti(III) ions with H₂O₂ and the subsequent precipitation with NH₄OH. We also have indications that the detected paramagnetic species may be complexed to the Ti(IV) ions. A striking feature in our results is the very high 2-year stability of these trapped O₂^{•−} radicals. We are able to confirm unequivocally the mechanism proposed by Haber and Weiss to explain the Fenton reagent reactions².

It is well known that free radicals are intermediates in many redox processes. The most important radical-forming redox reactions involve a metal ion that can undergo a one-electron transfer. Of these, the reaction of H₂O₂ with Fe(II) ions is the best known. Fenton discovered this reaction in 1894 and in 1932 Haber and Weiss³ proposed a mechanism that is now generally accepted.

It has been shown⁴ that if Fe(II) is replaced by Ti(III) the redox reactions between H₂O₂ and Ti(III) could be, in accordance with the postulated Haber–Weiss mechanism, formulated as:



and at higher H₂O₂: Ti(III) ratios



According to reaction (5) HO₂[•] and O₂^{•−} are in equilibrium and in strongly acidic solutions HO₂[•] should predominate. So far the formation in solution of the species O₂^{•−}, in measurable quantities has not been likely because of the strong acidity of the reaction system, thus there has been no report of detection by electron-spin resonance (ESR) of paramagnetic O₂^{•−} species generated from the redox reactions between Ti(III) ions and H₂O₂ in aqueous solutions. Other investigators^{4–6} have detected, by using fast-flow techniques, the generation of OH[•] and HO₂[•] radicals in the latter type of reactions. The formation of O₂^{•−}, O₂⁺ and O^{•−} species on the surface of TiO₂ was identified only in the case of vacuum-outgassed and oxygen-treated titanium dioxide^{7–10}.

In this work the titania gels were prepared by adding excess H₂O₂ (30 volumes) to a 15% titanous chloride solution and then adding a 1:1 ammonia solution until the required pH was

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1. Turco, R. P., Toon, O. B., Ackerman, T., Pollack, J. B. & Sagan, C. *Science* **222**, 1283–1291 (1983).
2. US National Research Council *The Effects on the Atmosphere of a Major Nuclear Exchange* (National Academy Press, Washington, DC, 1985).
3. Pittcock, A. B. *et al. Environmental Consequences of Nuclear War. Volume I. Physical and Atmospheric Effects* (Wiley, New York, 1986).
4. Thompson, S. L. *Nature* **317**, 35–39 (1985).
5. Malone, R. C., Auer, L. H., Glatzmaier, G. A., Wood, M. C. & Toon, O. B. *Science* **230**, 317–319 (1985).
6. Malone, R. C., Auer, L. H., Glatzmaier, G. A., Wood, M. C. & Toon, O. B. *J. geophys. Res.* **91**, 1039–1053 (1986).
7. Robock, A. *Nature* **310**, 667–670 (1984).
8. Covey, C., Schneider, S. H. & Thompson, S. L. *Nature* **308**, 21–25 (1984).
9. Covey, C., Thompson, S. L., & Schneider, S. H. *J. geophys. Res.* **90**, 5615–5628 (1985).

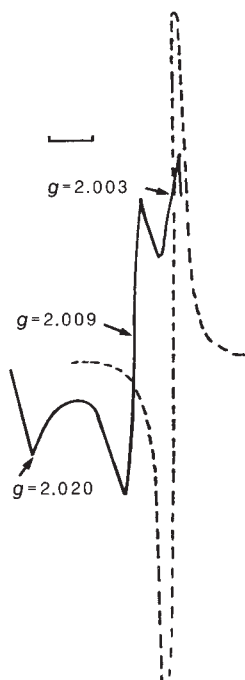
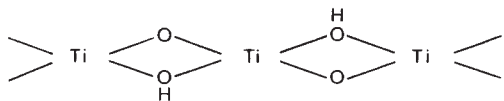


Fig. 1 ESR spectrum of colloid TiO_2 prepared at a pH of 6.9 and aged in distilled water for two years (dotted line, 1,1-diphenylpicrylhydrazyl (DPPH), $g = 2.0036$). Scale bar, 10 gauss.

attained. The gels were then filtered, washed free from chloride and dried in air at $\sim 110^\circ\text{C}$ for 24 h. Gels prepared under conditions of acidic pH values displayed, at room temperature, by ESR a triplet with 'g' values 2.003, 2.009 and 2.020 (Fig. 1) which, according to the literature^{8,10}, was identified as belonging to O_2^- on TiO_2 . It is interesting that, in this study, the ESR spectrum of such radicals did not escape detection. Indeed, in general for powders at room temperature, large anisotropy broadening occurs and is responsible for undetectable signals.

A very striking observation is that the triplet persisted after keeping the latter gels in air for 2 years. When the batches from which these gels were prepared were aged for 2 years in distilled water then heat-treated for 24 h at $\sim 110^\circ\text{C}$ in air, the same triplet appeared in the ESR spectrum. It has been shown^{11,12} that titania gels, prepared in a manner similar to the present one were formed of large polymeric entities resulting from crosslinking at intervals between these chains:



It therefore seems plausible to assume that it is this network structure that may be responsible for the bulk trapping of the free radicals and for the prevention of recombination.

Infrared studies indicate a broad band at $1,045\text{ cm}^{-1}$ for these gels characteristic of O_2^- radicals on TiO_2 (ref. 13) and a band at $1,389\text{ cm}^{-1}$ characteristic of the HO_2^- radicals¹⁴ (Fig. 2). Although in this study the latter radicals were detected by infrared spectroscopy, ESR studies did not give any indications of their existence. Chiang *et al.*⁶, investigating the reaction between Ti(III) chloride and H_2O_2 in acidic solutions, have shown that a doublet with 'g' values 2.0117 and 2.0102, is generally obtained with such systems. They identified the peaks as resulting from complexes involving $\text{OH}^\cdot$ and $\text{HO}_2^\cdot$ radicals and Ti(IV) , respectively, and showed that the formation of other radicals from this system actually took place 'within' the Ti(IV) complex. We did not observe such a doublet in our spectra; but it is possible that the 2.0102 line is superimposed on our observed triplet.

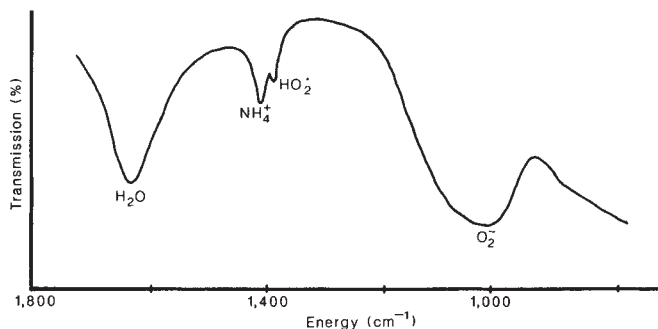


Fig. 2 Infrared spectrum measured on colloidal TiO_2 prepared at a pH of 2.5.

Gels prepared under neutral or alkaline pH values did not indicate, at room temperature in their ESR spectra, the existence of any paramagnetic species, but when the batches from which these gels were prepared were aged for 2 years in water and heat treated for 24 h in air, at $\sim 110^\circ\text{C}$ the triplet reappeared in the ESR spectrum. Infrared spectra measured on the unaged 'neutral' or 'high-pH' gels displayed peaks at 970 and $1,080\text{ cm}^{-1}$, which, according to the literature¹⁵, are attributed to bending modes of hydroxy complexes: the latter to a bending mode of a terminal (OH) and the former to a bending mode of a bridging (OH). No band was detected at $1,389\text{ cm}^{-1}$.

The disappearance of the triplet in the ESR spectrum of these gels is therefore accounted for by assuming that at high pH values, hydroxyl ions would totally replace the O_2^- species in the inner coordination sphere of the Ti ion. The reappearance of the O_2^- species after ageing in water could be explained if, on ageing, these free radicals (presumably near the Ti(IV) ion) diffuse back or regenerate into the inner coordination sphere of the titanium and reform a complex with the Ti(IV) ion. This suggestion is possible considering the non-rigid structure of the titania gel, which implies that the Ti(IV) is a component of the paramagnetic species giving the ESR signal.

Infrared spectra of the water-aged 'neutral' and 'high'-pH titania gels showed also regeneration of the $1,045\text{ cm}^{-1}$ band characteristic of the O_2^- species, but the $1,389\text{ cm}^{-1}$ band did not reappear in the latter case. These results give an unequivocal confirmation of the Haber-Weiss mechanism, according to which, in alkaline or neutral media the O_2^- species should predominate.

Henglein and co-workers¹⁶ developed techniques of probing and using the ability of colloidal TiO_2 to store electrons or holes, which were used to reduce or oxidize compounds in solution. The absorption spectra of the long lived excess of electrons and positive holes trapped at the interface were measured by flash photolysis.

In a series of controlled experiments, titania gels prepared at different pH values from TiCl_3 and NH_4OH with oxygen gas as oxidizing agent, preparation described previously¹², did not display by ESR any observable signals, thus providing further evidence that the detected paramagnetic species originate from the redox reaction between H_2O_2 and Ti(III) . Also, infrared studies carried out on these gels did not display the $1,389\text{ cm}^{-1}$ and $1,045\text{ cm}^{-1}$ bands.

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1. Symons, M. C. R. & McLauchlan, K. A. *Faraday Discuss. chem. Soc.* **78**, 7-17 (1984).
2. Uri, N. *Chem. Rev.* **50**, 375-454 (1952).
3. Pryor, W. A. in *Free Radicals*, 136-137 (McGraw-Hill, London, 1966).
4. Takakura, K. & Ranby, B. *J. phys. Chem.* **72**, 164-168 (1968).
5. Nixon, W. T. & Norman, R. O. C. *Nature* **196**, 891-892 (1962).
6. Chiang, Y. S., Craddock, J., Mickewich, D. & Turkevich, J. *J. chem. Phys.* **70**, 3509-3515 (1966).

7. Lunford, J. H. & Jayne, J. P. *J. chem. Phys.* **44**, 1487-1492 (1966).
8. Naccache, C., Meriaudeau, P., Che, M. & Tench, A. J. *Trans. Faraday Soc.* **67**, 506-512 (1971).
9. Dufaux, M., Che, M. & Naccache, C. *C.r. heb. Séanc. Acad. Sci., Paris* **258**, 2255-2267 (1969).
10. Iyengar, R. D. & Kellermann, R. K. *J. Colloid Sci.* **35**, 424-433 (1971).
11. Ragai, J. & Sing, K. S. W. *J. Colloid Sci.* **101**, 369-376 (1984).
12. Ragai, J. & Selim, S. I. *J. Colloid Sci.* (in the press).
13. Gundrizer, G. A. *Mater. Konf. Molodykh Uch. Tomsk. gos. Univ.* **2**, 166-168 (1974).
14. Milligan, D. F. & Jacox, M. E. *J. chem. Phys.* **38**, 2627-2631 (1963).
15. Nakamoto, K. in *Infrared and Raman Spectra of Inorganic and Coordination Compounds*, 229-230 (Wiley, New York, 1978).
16. Bahnmann, D., Henglein, A. & Lubomir, S. *Faraday Discuss. chem. Soc.* **78**, 151-163 (1984).

Mechanism of diopside dissolution from hydrogen depth profiling

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The basic processes involved at the reaction interface during the aqueous dissolution of crystalline silicates are a matter of controversy. In particular, the existence of a hydrated or protonated surficial layer which has been invoked to explain dissolution kinetics has not been demonstrated yet. By using a resonant nuclear reaction, which allows hydrogen depth profiling, we present, in the case of diopside, the first direct evidence that dissolution proceeds via a surficial hydration over thicknesses of about 1,000 Å. This evidence is combined with X-ray photoelectron spectrometry and secondary ion mass spectrometry data, to show that approximately congruent leaching at energetic portions of the mineral surface allows permeation of the resulting porous structure by water, much of which is strongly bonded to the silicate surface. Thus, we suggest that the migration of molecular water into the surface could be a key step in the dissolution kinetics of diopside and probably of other cationic silicate minerals.

The aqueous dissolution of minerals has been a subject of intensive investigation for more than 25 years because of its importance in Earth sciences. In fact, water-rock interactions have important implications in various fields such as weathering which is a key step in the geochemical cycles of elements such as silicon, aluminium, magnesium and iron, ore forming processes. However, the basic processes involved at the reaction interface, particularly for silicates, are still a matter of controversy. Among the models proposed, essentially two main mechanisms can be distinguished, depending on whether the rate determining step is a transport step, for example, a transport of reactant through an altered surficial layer¹⁻³ or the dissolution rate is controlled by a surface reaction⁴⁻⁸. In this latter theory, the rate limiting step for dissolution has been attributed to the irreversible decomposition of a surficial protonated activated complex, the stoichiometry of which depends to a large extent on the crystallochemical properties of the mineral at the surface active sites^{5,7,8}. This theory is now widely accepted, as a chemically altered layer has not been seen on mineral surfaces dissolved in natural or laboratory conditions^{5,6,9}, either by direct scanning electron microscopy observations or through chemical analyses with X-ray photoelectron spectrometry (XPS). Also, in support of the surface control of reaction rates, recent studies have pinpointed the important role of surface defects (for example, point defects, dislocations) on the dissolution kinetics^{6,9,10,11}. However, the existence of a protonated material, at the very

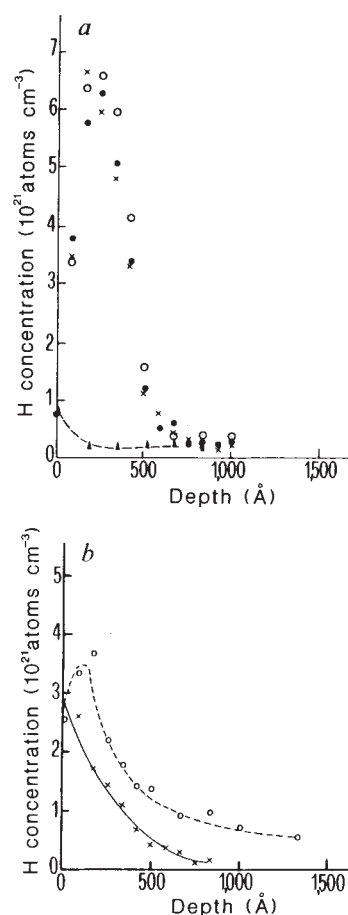


Fig. 1 Evidence for hydrogen penetration in leached diopside. *a* shows RNR H-depth profiles before and after leaching in various conditions $\blacktriangle$, diopside 1 untreated; $\circ$, diopside 1 leached for 75 days at 25 °C and pH 2; $\bullet$, diopside 1 leached for 75 days at 25 °C and pH 6; $\times$, diopside 2 leached for 75 days at 25 °C and pH 2. *b*, The effect of a one hour post-leaching thermal treatment at 150 °C on the RNR H-profile of sample no. 1. $\circ$, Diopside 1 leached; $\times$, diopside 1 leached and treated for an hour at 150 °C.

surface of the solid^{5,10,11}, has been postulated on indirect evidence derived from chemical analysis of elements other than hydrogen but has never been directly proved.

In this work, we have applied an analytical technique based on a specific resonant nuclear reaction (RNR) which allows a direct depth profiling of hydrogen on near-surface regions in order to untangle this question. This technique has already been applied successfully to silicate glasses^{14,15} and more recently to ion-bombarded minerals¹⁶. We have chosen diopside because a recent and detailed XPS study⁵ has been performed on this mineral, and because there was a reasonable chance of proving whether a hydrated layer was present on the mineral surface during aqueous dissolution. In the first step of this investigation, we concentrated our efforts on the chemical characterization of the mineral surface after dissolution, as it seemed to us important to show unambiguously the presence of a hydrated layer on dissolved diopside.

Centimetre-sized polished sections of two monocrystalline diopsides (sample no. 1 from Rothenkopf (FRG), and sample no. 2 from Val d'Ala (Italy)) were subjected to dissolution in buffered aqueous solutions placed in teflon bottles (pH 2 and 6) at 25, 51 and 100 °C during periods ranging from 5 to 75 days, and with a surface area to solution volume ratio $\sim 2 \times 10^{-3} \text{ cm}^{-1}$. The samples were cut along the c-axis and polished very carefully to produce a flat surface necessary for proper analysis; in this procedure we have tried to minimize defect

production by using fine-grained polishing. In addition, to avoid hydration during polishing we used ethanol or ethylene glycol as a paste solvent instead of water. The buffers used in these experiments were mixtures of 0.2 mol l^{-1} KCl and 0.2 mol l^{-1} HCl (pH 2) and of 0.1 mol l^{-1} K-hydrogen phthalate and 0.1 mol l^{-1} NaOH aqueous solutions (pH 6). The chemical analyses of the diopside crystals were performed with a Camebax electron microprobe and are, for samples 1 and 2 respectively: $\text{Ca}_{.98}\text{Mg}_{.93}\text{Fe}_{.07}\text{Si}_2\text{O}_6$ and $\text{Ca}_{.98}\text{Mg}_{.97}\text{Fe}_{.045}\text{Si}_2\text{O}_6$. The H-depth profiles were obtained with the $^1\text{H}(^{15}\text{N}, \alpha\gamma)^{12}\text{C}$ nuclear reaction which presents a resonance at 6.385 MeV and allows a resolution of $\sim 30\text{--}100 \text{ \AA}$. The principles of this technique are presented in ref. 14 and our particular operating conditions are described in ref. 15. Before each run we made the H-profile of a reference allowing a quantitative evaluation of the hydrogen concentration; this reference consists of a monocrystal of silicon implanted with a given dose of 30 keV hydrogen ions. We observed also that the experimental H-profile reproduces perfectly the expected theoretical distribution of implanted hydrogen with the exact range. In order to avoid any charging effect, the samples were covered with a $\sim 100 \text{ \AA}$ aluminium coating. The results obtained are described below.

Before any dissolution, diopside samples exhibit very limited H penetration (Fig. 1a). The H-profile shows a maximum at the very surface $\leq 10^{21} \text{ H cm}^{-3}$, that is, ≤ 1 atom %, and extends to a depth $< 200 \text{ \AA}$. A similar profile has been obtained with diopside sample no. 2. The corresponding surficial amount of hydrogen is probably due to a contamination linked to sample preparation, residual hydrocarbons in the vacuum chamber or contact of samples with atmospheric moisture. After dissolution (up to 75 days) by contrast, one notes a marked H penetration in the crystals (Fig. 1a). The profiles are almost identical for the two diopsides and the two pH s. The shape of the profiles is quite unexpected as they exhibit a sharp maximum beneath the surface, the maximum hydrogen concentration observed being $\sim 6.6 \times 10^{21} \text{ H cm}^{-3}$ at around $250 \text{ \AA}$ (~ 8.5 atom %) for samples leached for 75 days. Moreover, the hydrogen profile extends to $\sim 700 \text{ \AA}$ from the surface of the mineral. These H-profiles were reproducible after sample storage up to one year as well as stable under the $^{15}\text{N}^+$ beam, even after one half hour bombardment on the same spot.

These results represent the first direct evidence for an important H penetration in a crystalline silicate during its aqueous dissolution. However, our technique does not allow by itself any discrimination between hydrogen containing species (H^+ , H_3O^+ or H_2O).

In order to further characterize the chemical form of hydrogen, one sample of diopside no. 1, has been heated for one hour at 150°C before analysis. The resulting RNR profile (Fig. 1b) shows a partial loss of hydrogen of $\sim 25\%$. This feature indicates that hydrogen is probably partly in the form of molecular water, easily released during thermal treatment, and also, as a species, strongly bonded to the silicate network. This combination of labile and bonded forms of hydrogen could also explain the peculiar peaked shape of the profiles similar to those observed with silicate glasses¹⁷, because the pumping of the sample in the vacuum chamber would have partially removed water from the surface-most zones resulting in a sub-surface maximum. In addition, we have investigated the effect of various parameters on the dissolution characteristics: (1) with increasing temperature, the general trend is that the total amount of penetrating hydrogen decreases (Fig. 2a). For example, after 30 days at 51°C the maximum of hydrogen is $\sim 2 \times 10^{21} \text{ H cm}^{-3}$ (~ 3 atom %), although it corresponds to a greater depth $\sim 500 \text{ \AA}$, and is even smaller at 100°C after 8 days of leaching. Such an observation suggests uptake of H_2O as opposed to H^+ , with decreasing H_2O uptake with increasing temperature explained by decreasing surface hydration. (2) There is surprisingly no marked pH dependence in the amplitude of H penetration. For instance, diopside no. 1 leached 30 days at 51°C shows very similar

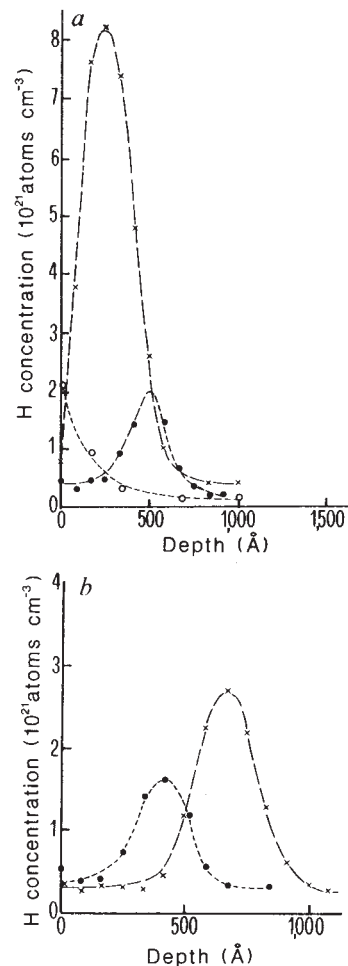


Fig. 2 Panel a shows the temperature dependence at pH 6 for sample no. 1 leached, $\times$, 75 days at 25°C $\bullet$, 30 days at 51°C and $\circ$, 8 days at 100°C . b, The effect of pre-etching with a $\text{HF} + \text{H}_2\text{SO}_4$ solution on sample no. 2 subsequently leached, $\times$, 30 days at 51°C and pH 2 compared to $\bullet$, the reference sample normally leached under the same conditions.

H-profiles at both pH 2 and 6 (Fig. 1a). This also tends to support the penetration of hydrogen into the mineral in the form of molecular water even in acidic solutions and not only as H^+ ions replacing cations within the crystal as suggested by the protonated 'surface layer' theories. Similar conclusions have been reached for silicate glasses^{17,18}. (3) We have also pre-treated the sample surface with a $\text{HF} + \text{H}_2\text{SO}_4$ solution at 25°C . By this process, dislocations or fissures originally present or possibly induced by the polishing and preparation technique⁵ are enlarged. With HF treatment we find that H penetration seems to be slightly higher than without any HF treatment, the maximum H-concentration and depth being $2.8 \times 10^{21} \text{ H cm}^{-3}$ and $600 \text{ \AA}$ instead of $1.6 \times 10^{21} \text{ H cm}^{-3}$ and $400 \text{ \AA}$, respectively (Fig. 2b). This is to be expected if the HF treatment produces tiny 'holes' in the surface which are filled with water. In addition, the samples have been carefully examined with both an optical microscope and a SEM and we do not observe any detectable fissuring of the untreated surfaces. Thus the 'holes' result probably from the enlargement of original dislocations by deep etch pit formation.

Data on elements other than hydrogen were obtained by XPS and secondary ion mass spectrometry (SIMS) which allow surface analyses and depth profiles of constituent elements of solids. By using XPS, it was found^{5,13} that during laboratory dissolution of diopside at $pH \sim 2\text{--}6$ a very thin Ca-depleted zone, on the scale of a few atomic layers ($5\text{--}15 \text{ \AA}$), formed very early on the

surface (during the first few hours) but did not increase in thickness as the experiment continued (up to 40 days). This was explained by a stoichiometric dissolution of diopside after a short initial incongruent reaction. Complementary data on elemental depth profiles by SIMS, which will be reported elsewhere, are compatible with XPS results in showing a decrease in the signal of all elements after leaching, along with a preservation of diopside original stoichiometry. This decrease in all elements towards the surface is believed to indicate an increase in the porosity of the surface, the pore space of which, on the basis of RNR results, is filled with water.

There is therefore clear evidence for the presence, during dissolution of diopside, of important quantities of hydrogen probably as H_2O up to a depth of $\sim 1,000 \text{ \AA}$ coupled with a notable depletion in all elements signifying an increase in porosity over this thickness. Most of this water is strongly bonded within the pores as attested to by its presence after being subjected to high vacuum, ion bombardments and even heating for one hour at 150°C .

Our results do not agree with the idea that the surface of diopside is represented by a newly-precipitated hydrated phase. Such a precipitate model could indeed explain the existence of a thick hydrogen-containing phase on the surface, but it is incompatible with the H-profiles that we have observed, as this phase should have a constant hydrogen concentration with depth. Also, one would not expect to find essential Ca-Mg-Si stoichiometry as suggested by our XPS data.

Likewise, when one compares the variations with depth of the concentrations obtained by RNR, SIMS and XPS on leached diopside, one concludes that there is no simple correlation between the penetration of hydrogen into this mineral during dissolution and the remobilization of constituent elements. Therefore, our results cannot be accounted for by a simple ionic diffusion or interdiffusion model neither through a leached residual layer or through a reprecipitated one. This result is markedly different from the case of simple silicate glasses for which a ratio between hydrogen and network modifiers ~ 1 or 3 has been measured, leading investigators^{14,15,19} to propose a leach model based on an H^+ (or H_3O^+)- Na^+ (or alkali⁺) interdiffusion.

The absence of a direct correlation between hydrogen penetration and cation release, as well as the behaviour of leached samples upon heating, also supports the hypothesis of molecular water permeation as already postulated for silicate glasses^{17,20}. Diffusion of molecular water and its reaction with the silicate network could henceforth be an important limiting step for the kinetics of silicate dissolution. The density of defects (especially dislocation cores) should play a major role in the migration of water by permitting water molecules to jump from one interstice to another²¹. Simultaneously, the leaching and hydrolysis of the silicate network would 'open the structure' until complete destruction and would enhance the penetration of water inside the mineral. The reaction of water with the network would be responsible for the strong bonding of a fraction of hydrogen and its retention. Our data suggest the formation at the surface of leached diopside of a porous hydrated silicate depleted almost congruently in all elements and therefore of much lighter density. This conclusion is compatible with previous experimental results^{5,9} on diopside and other silicates (albite, enstatite, bronzite and tremolite), which indicate essentially congruent dissolution behavior and a stoichiometric composition of solids below the outermost $\sim 10 \text{ \AA}$. It is also in agreement with the suggestion of Berner *et al.*²² that the surface of dissolving silicates is porous because of dissolution along dislocations and microcracks, for example.

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1. Wollast, R. *Geochim. cosmochim. Acta* **31**, 635-648 (1967).
2. Helgeson, H. C. *Geochim. cosmochim. Acta* **35**, 421-469 (1971).
3. Paces, T. *Geochim. cosmochim. Acta* **37**, 2641-2663 (1973).
4. Lagache, M. *Bull. Soc. fr. Minér. Cristallogr.* **88**, 223-253 (1965).
5. Schott, J., Berner, R. A. & Sjöberg, E. L. *Geochim. cosmochim. Acta* **45**, 2123-2135 (1981).
6. Holdren, G. R. & Berner, R. A. *Geochim. cosmochim. Acta* **43**, 1161-1171 (1979).
7. Aagaard, P. & Helgeson, H. C. *Am. J. Sci.* **282**, 237-285 (1982).
8. Helgeson, H. C., Murphy, W. M. & Aagaard, P. *Geochim. cosmochim. Acta* **48**, 2405-2432 (1984).
9. Berner, R. A. & Holdren, G. R. *Geochim. cosmochim. Acta* **43**, 1173-1186 (1979).
10. Brantley, S. L., Crane, S. R., Crerar, D. A., Hellmann, R. & Stallard, R. *Geochim. cosmochim. Acta* **50**, 2349-2361 (1986).
11. Lasaga, A. C. & Blum, A. E. *Geochim. cosmochim. Acta* **50**, 2363-2379 (1986).
12. Chou, L. & Wollast, R. *Geochim. cosmochim. Acta* **48**, 2205-2217 (1984).
13. Schott, J. & Berner, R. A. *Geochim. cosmochim. Acta* **47**, 2333-2340 (1983).
14. Lanford, W. A., Davis, K., Lamarche, P., Laursen, T. & Groleau, R. J. *J. Non-Cryst. Solids* **33**, 249-266 (1979).
15. Della Mea, G., Dran, J.-C., Petit, J.-C., Bezzon, G. & Rossi-Alvarez, C., *Nucl. Instrum. Meth.* **218**, 493-499 (1983).
16. Petit, J.-C., Dran, J.-C. & Della Mea, G., *Bull. Minér.* **110**, 25-42 (1987).
17. Bunker, B. C., Arnold, G. W., Beauchamp, E. K. & Day, D. E. *J. Non-Cryst. Solids* **58**, 295-322 (1983).
18. Smets, B. M. L. & Tholen, M. G. W. *Phys. Chem. Glasses* **26**, 60-63 (1985).
19. Doremus, R. H., *J. non-cryst. Solids* **19**, 137-144 (1975).
20. Smets, B. M. L. & Lommen, T. P. A. *Phys. Chem. Glasses* **23**, 83-87 (1982).
21. Dran, J. C., Langevin, Y. & Petit, J. C. *Nucl. Instrum. Meth.* **B1**, 557-568 (1984).
22. Berner, R. A., Schott, J. & Holdren, G. R. *Geochim. cosmochim. Acta* **49**, 1657-1658 (1985).

Processes regulating sulphate flux after whole-tree harvesting

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Terrestrial processes that regulate transfer of strong-acid anions (for example SO_4^{2-} , NO_3^- , Cl^-) play an important role in determining the acid-base status of surface waters¹. Anthropogenic perturbations of forested watersheds can alter these processes, resulting in changes of surface-water chemistry^{2,3}. Much controversy has arisen over the relative importance of acidic deposition, natural processes of soil acidification and the effects of changes in land use on the acidification of surface waters^{4,5,6}. Forest clearcutting represents a useful experimental tool to evaluate the effects of changes in strong-acid loading on biogeochemical processes controlling SO_4^{2-} retention and release. Here we report that after the whole-tree harvesting of an experimental watershed at the Hubbard Brook Experimental Forest (HBEF) in the White Mountains of New Hampshire, USA, increased mineralization and nitrification led to substantial NO_3^- loss, acidification of soil solutions and increased soil adsorption of SO_4^{2-} . As a consequence, solution concentrations and streamwater efflux of SO_4^{2-} declined. Substantial increases in streamwater concentrations of H^+ and potentially toxic inorganic Al^{3+} after removal of biomass also occurred. A similar disruption of the soil N cycle observed in areas of forest decline^{7,8} suggests that decreased vegetation uptake of N may adversely affect surface water quality in acid-sensitive regions.

Previous research at the HBEF has demonstrated the importance of ecosystem processes in nutrient retention. After revegetation of watershed 2 (W2) at the HBEF in 1965, a substantial increase in the efflux of NO_3^- , H^+ , Al^{3+} and basic cations (Ca^{2+} , Mg^{2+} , K^+ and Na^+) occurred with streamflow. In these experi-

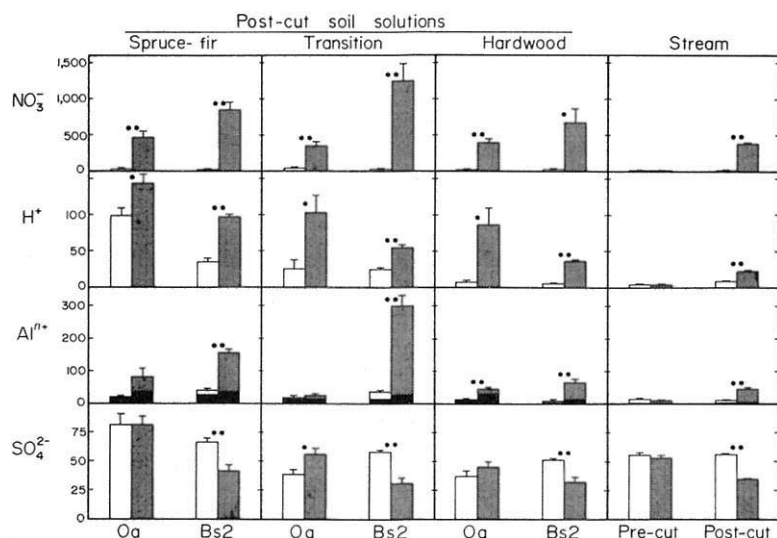


Fig. 1 Soil solution and stream concentrations ($\mu\text{mol l}^{-1}$) after whole-tree harvesting of watershed 5 (shaded bars) and the reference watershed 6 (clear bars). Darkened (lower) portions of the Al bars represent non-labile monomeric (organically complexed) Al, and the top portion labile monomeric (inorganic) $\text{Al}^{20,21}$. Post-cut solutions were collected between 20 November 1984 and 15 March 1985, the dormant period before snowmelt when hydrological fluxes are unaffected by transpiration. Stream solutions before cutting were sampled during the same period in 1983–84, before any clearcut effects. Asterisks denote significant differences between means at the $P < 0.05$ (*) and $P < 0.01$ (**) levels.

ments, conventional commercial clearcutting practices were not used. Rather, trees were felled, left in place, and herbicides applied to prevent vegetation regrowth. These treatments were designed to separate the effects of vegetation uptake from increased decomposition on the mobilization of dissolved nutrients^{9,10}.

Although acidification and basic cation mobilization were evident, both concentration and efflux of stream SO_4^{2-} declined for the three years of herbicide treatment afterwards. The onset of vegetation regrowth after the cessation of herbicide treatment coincided with a decrease in the acidity of drainage water and an increase in the concentration and transport of SO_4^{2-} . A number of alternative hypotheses could explain the decrease in stream SO_4^{2-} concentrations after revegetation: H1: herbicide application selectively altered soil S transformations; H2: loss of foliar surface area decreased inputs of dry deposition¹¹; H3: decomposition of forest-floor organic matter increased, enhancing microbial immobilization of SO_4^{2-} into organic forms¹², or inhibiting organic S mineralization¹⁰; H4: decreased transpiration resulted in increased hydrological flux and dilution of stream SO_4^{2-} concentrations¹⁰; H5: sulphate was reduced to gaseous (for example H_2S) or solid (FeS) phases¹⁰; H6: sulphate

was retained owing to precipitation of aluminium hydroxysulphate minerals, such as jurbanite ($\text{Al}(\text{OH})\text{SO}_4 \cdot 5\text{H}_2\text{O}$); H7: adsorption of SO_4^{2-} to free Al and/or Fe oxide surfaces increased owing to acidification of soil solutions¹³.

To test these hypotheses, a watershed manipulation experiment was done at the HBEF. From autumn 1983 through spring 1984 an experimental watershed (W5) underwent a commercial whole-tree harvest involving the removal of all aboveground tree biomass. This manipulation was evaluated using the paired watershed approach, in which the experimental watershed and an adjacent untreated reference watershed (W6) were monitored. Both watersheds were instrumented with stream gauging weirs, bulk precipitation and throughfall collectors, and 'tension-free' soil lysimeters¹⁴ beneath the Oa (forest floor) and within Bs2 horizons above impervious schist bedrock. Watersheds 5 and 6 are dominated largely by northern hardwood forest vegetation, consisting of yellow birch (*Betula alleghaniensis*), American beech (*Fagus grandifolia*) and sugar maple (*Acer saccharum*). A spruce-fir zone at the top of each watershed is dominated by red spruce (*Picea rubens*) and balsam fir (*Abies balsamea*). Elevational differences in vegetation and soil depth contribute to substantial spatial variability in the chemistry of drainage waters in these catchments^{15,16}. Consequently, replicate lysimeters were installed in a low-elevation hardwood zone (600 m; $n = 3$) near the base of each watershed, in a hardwood transition zone (730 m; $n = 3$) and in a high-elevation spruce-fir zone (750 m; $n = 2$) near the top of each watershed.

After whole-tree harvesting of W5, patterns of element concentration and transport (Fig. 1) were similar to those after revegetation of W2. Increased concentrations of NO_3^- were released from soil to stream solutions owing to reduced vegetative uptake coupled with increased mineralization and nitrification caused by higher soil moisture and temperature^{7,17}. Mineralization and nitrification of soil organic N produces one mole of H^+ for each mole of NO_3^- ; however, increases in H^+ concentration were not stoichiometric with respect to NO_3^- release. Rather, increased concentrations of basic cations and Al were also leached from both organic and mineral soil horizons owing to a combination of mineralization, exchange and dissolution reactions. Increases in Al concentration largely occurred as the labile (inorganic) monomeric form, which has been shown to be toxic to aquatic organisms^{18,19}. Concentrations of SO_4^{2-} declined significantly ($P < 0.01$) after the whole-tree harvest (Fig. 1) in both lower-mineral soil solutions (W5, Bs2) and streamwater. Beneath the forest floor (Oa), however, SO_4^{2-} concentrations remained stable or significantly increased after the manipulation.

Patterns of solute release enabled us to test the research

Table 1 Saturation indices (SI) for lower mineral soil (Bs2) and stream solutions from November 1984 to March 1985 in W6 (reference watershed) and W5 (whole-tree harvested watershed)

Site	Watershed	Saturation index	
		Jurbanite	Gibbsite
Spruce-fir* (750 m)	W6 soil	-0.99	-0.40
	W6 stream	-1.38	-1.54
	W5 soil	-0.78	-0.85
	W5 stream	-0.99	0.20
Transition (730 m)	W6 soil	-0.75	0.14
	W6 stream	-0.78	-0.11
	W5 soil	-0.58	0.17
	W5 stream	-0.79	-0.51
Hardwood (600 m)	W6 soil	-1.87	0.54
	W6 stream	-1.13	0.28
	W5 soil	-0.87	0.01
	W5 stream	-0.82	0.21

$\text{SI} = \log (Q_p / K_p)$, where Q_p is the ion activity product and K_p is the equilibrium constant. Positive values indicate oversaturation, negative values undersaturation with respect to the mineral phase of interest.

*Spruce-fir soil and stream solutions are typically undersaturated with respect to all mineral phases, owing to a combination of shallow soils (short hydrologic residence time), low pH and high dissolved organic C concentration, all of which favour a kinetic restraint on attaining saturation.

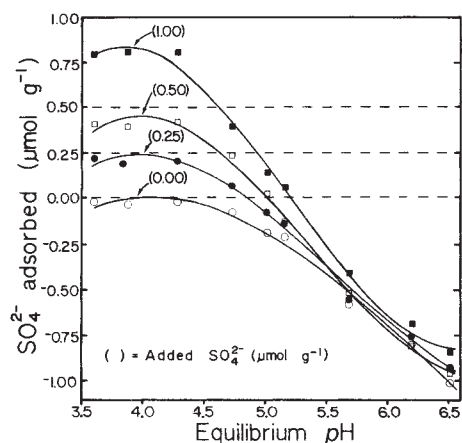


Fig. 2 Sulphate adsorbed by HBEF Bs2 horizon soil at different concentrations of added sulphate across a range of equilibrium solution pHs. Negative adsorption numbers are due to desorption of ambient sulphate. Lines represent the best fit of a third-order polynomial equation.

hypotheses (H1-H7) posed to explain decreases in the SO_4^{2-} concentration of drainage water after forest removal. As herbicides were not applied in the experiment, a selective action of the herbicide on S transformations was not possible (H1). Decreases in dry deposition inputs to the canopy as a result of canopy loss (H2), increases or decreases in S immobilization or mineralization rates, respectively, in the forest floor (H3), and dilution of SO_4^{2-} due to increased hydrologic flux (H4) would all be expected to result in decreased concentrations of SO_4^{2-} in Oa-horizon leachates. Consequently these hypotheses can be rejected as the proximate cause of decreased SO_4^{2-} concentrations in mineral soil and stream solutions after clearcutting. Decreases in SO_4^{2-} concentration due to reduced dry deposition of S were probably compensated for by increased mineralization of organic S to SO_4^{2-} in the forest floor¹². Microbial reduction of SO_4^{2-} to S^{2-} (H5) is also unlikely, owing to the high concentrations of NO_3^- in soil solution and the fact that of NO_3^- reduction is more energetically favourable²² and would proceed before SO_4^{2-} reduction.

Possible control of solution SO_4^{2-} concentrations by jurbanite ($\text{Al}(\text{OH})\text{SO}_4 \cdot 5\text{H}_2\text{O}$) precipitation (H6) was investigated by calculating mineral saturation indices (Table 1) for jurbanite and natural gibbsite using the chemical equilibrium model ALCHEMI²³. This computer model calculates the distribution of inorganic Al complexes, adjusting for temperature and ionic strength effects²³. Soil and stream solutions were undersaturated with respect to the solubility of jurbanite in both watersheds at all elevations (Table 1), suggesting that jurbanite precipitation was not thermodynamically favourable and probably not responsible for the decline in solution SO_4^{2-} concentrations after the whole-tree harvest. Soil and stream solutions in the transition and low-elevation zones were closer to saturation with respect to the solubility of natural gibbsite, or a mineral of similar solubility. Although the solubility of $\text{Al}(\text{OH})\text{SO}_4 \cdot 5\text{H}_2\text{O}$ apparently does not control SO_4^{2-} concentrations at the HBEF, this process is considered important in regions with high atmospheric inputs of SO_4^{2-} , such as parts of the Federal Republic of Germany and The Netherlands^{24,25}.

Adsorption of SO_4^{2-} onto free sesquioxide surfaces (H7) was investigated using batch titration studies in which Na_2SO_4 was added to suspensions of Bs2 mineral horizon soil (taken from 30 soil pits in W5) with solution pH adjusted between 3.5 and 6.5 with HNO_3 or NaOH . After centrifugation, pH and soluble SO_4^{2-} were measured and adsorbed SO_4^{2-} was calculated from the amount removed from or added to solution. Acidification of the soil solution greatly enhanced retention of SO_4^{2-} (Fig. 2). In the range of pH values experienced by lower mineral soil

solutions at the HBEF (4.5 to 5.3), SO_4^{2-} adsorption is highly dependent on pH²⁶. As pH decreases to 4.3, the extent of SO_4^{2-} adsorption increases owing to protonation of variable-charge surfaces such as $\text{Al}(\text{OH})_3$ or $\text{Fe}(\text{OH})_3$. Below pH 4.3, the extent of SO_4^{2-} adsorption diminishes. We attribute this decrease to dissolution of soil Al, which forms soluble Al-SO_4^+ complexes which compete with the adsorbent phase for SO_4^{2-} .

On the basis of laboratory and field soil solution monitoring studies, all of the hypotheses advanced to explain the reductions in stream SO_4^{2-} concentrations after whole-tree harvesting can be rejected with the exception of acidification-induced SO_4^{2-} adsorption in lower mineral soil horizons. Furthermore, adsorption appears to be an important factor regulating seasonal variability in stream SO_4^{2-} concentrations in undisturbed watersheds²⁷. Regression analysis of stream SO_4^{2-} and H^+ concentrations (μ equiv. l^{-1}) in the reference watershed over a 2.5 year period yielded a significant inverse relationship ($[\text{SO}_4^{2-}] = 145 - 3.25 [\text{H}^+]$; $r^2 = 0.49$; $P < 0.0001$; $n = 31$), consistent with the pH-dependent SO_4^{2-} adsorption hypothesis.

Current models developed to simulate the response of forest ecosystems to strong-acid inputs generally fail to consider pH-dependent SO_4^{2-} adsorption²⁸⁻³¹. Results of this whole-tree harvest experiment indicate that strong-acid inputs, even though they may change soil solution pH only slightly, can facilitate SO_4^{2-} retention, which buffers surface water acidification. Therefore simulation models that fail to account for pH-dependent SO_4^{2-} adsorption may overestimate the consequences of increased inputs of strong acids to waters draining forest ecosystems.

The whole-tree harvest experiment also demonstrated that disruption of the soil N cycle resulting from biomass removal can cause short-term acidification of soil solutions and streams and the release of toxic inorganic Al to surface waters¹⁸. In view of current concerns about forest decline³², a link between forest degradation and surface-water acidification is especially noteworthy. If forest decline is accompanied by substantial NO_3^- efflux, as has been observed in the Federal Republic of Germany⁷ and Czechoslovakia⁸, then the potential for damage to surface-water quality is present.

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1. van Breeman, N., Mulder, J. & Driscoll, C. T. *Pl. Soil* **75**, 283-308 (1983).
2. Bormann, F. H. & Likens, G. E. *Pattern and Process in a Forested Ecosystem* (Springer, New York, 1981).
3. Vitousek, P. M. *et al. Science* **204**, 469-474 (1979).
4. Krug, E. C. & Frink, C. R. *Science* **221**, 520-525 (1983).
5. Glover, G. M. & Webb, A. H. *Water Res.* **13**, 661-667 (1979).
6. Driscoll, C. T. & Newton, R. M. *Envir. Sci. Technol.* **19**, 1018-1024 (1985).
7. Ulrich, B. in *Effects of Accumulation of Air Pollutants in Forest Ecosystems* (eds Ulrich, B. & Pankrath, J.) 1-29 (Reidel, Dordrecht, 1983).
8. Paces, T. *Nature* **315**, 31-36 (1985).
9. Likens, G. E., Bormann, F. H., Johnson, N. M., Fisher, D. W. & Pierce, R. S. *Ecol. Monogr.* **40**, 23-47 (1970).
10. Likens, G. E., Bormann, F. H., Pierce, R. S. & Reiniers, W. A. *Science* **199**, 492-496 (1978).
11. Hultberg, H. *Ecol. Bull. (Stockholm)* **3** (in the press).
12. David, M. B., Mitchell, M. J. & Schindler, S. C. in *Forest Soils and Treatment Impacts* (Proc. Sixth N. Am. Forest Soils Conf.) (ed. E. L. Stone) 221-245 (Univ. Tenn., Knoxville, 1984).
13. Davis, J. A. & Leckie, J. O. *J. Colloid Interface Sci.* **74**, 32-43 (1980).
14. Turner, R. S., Johnson, A. H. & Wang, D. *J. envir. Qual.* **14**, 314-323 (1985).
15. Johnson, N. M., Driscoll, C. T., Eaton, J. S., Likens, G. E. & McDowell, W. H. *Geochim. cosmochim. Acta* **45**, 1421-1437 (1981).
16. Lawrence, G. B., Fuller, R. D. & Driscoll, C. T. *Biogeochemistry* **2**, 115-135 (1986).
17. Smith, W. H., Bormann, F. H. & Likens, G. E. *Soil Sci.* **106**, 471-473 (1968).
18. Baker, J. P. & Schofield, C. L. *Wat. Air Soil Pollut.* **18**, 289-309 (1982).
19. Hall, R. J., Driscoll, C. T., Likens, G. E. & Pratt, M. J. *Limnol. Oceanogr.* **30**, 212-220 (1985).
20. Barnes, R. B. *Chem. Geol.* **15**, 177-191 (1975).
21. Driscoll, C. T. *Int. J. envir. Anal. Chem.* **16**, 267-284 (1984).
22. Stumm, W. & Morgan, J. J. *Aquatic Chemistry* (Wiley, New York, 1981).
23. Schecher, W. D. & Driscoll, C. T. *Wat. Resour. Res.* (in the press).

24. Prenzel, J. in *Effects of Accumulation of Air Pollutants in Forest Ecosystems* (eds Ulrich, B. & Pankrath, J.) 157–170 (Reidel, Dordrecht, 1983).
25. van Breemen, N. *Proc. Soil Sci. Soc. Am.* **37**, 694–697 (1973).
26. Nodvin, S. C., Driscoll, C. T. & Likens, G. E. *Soil Sci.* **142**, 69–75 (1986).
27. Nodvin, S. C. thesis (Cornell Univ., New York, 1983).
28. Christopherson, N. & Wright, R. F. *Wat. Resour. Res.* **17**, 377–389 (1981).
29. Schnoor, J. L., Palmer, W. D. & Glass, G. E. in *Modeling of Total Acid Precipitation Impacts* (ed. Schnoor, J. L.) 155–173 (Butterworth, Boston, 1984).
30. Cosby, B. J., Wright, R. F., Hornberger, G. M. & Galloway, J. N. *Wat. Resour. Res.* **21**, 1591–1601 (1985).
31. Gherini, S. A. *et al.* *Wat. Air Soil Pollut.* **26**, 425–459.
32. Johnson, A. H. & Siccama, T. G. *Envir. Sci. Technol.* **17**, 294A–305A (1983).

High rates of consumption of bacteria by pelagic ciliates

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It has been thought that ciliate protozoans that consume bacteria are not found in open waters except at micro-sites of high bacterial density^{1,2}. Previous laboratory studies, using ciliates isolated from benthic habitats have suggested that (1), ciliates that can ingest bacterial-sized particles have cell-specific clearance rates too low to obtain sufficient food for growth at the bacterial abundances found in most marine and fresh waters^{3,4}, and (2), spirotrichous ciliates cannot ingest particles of less than 1–2 μm in diameter^{3,4}. New data on uptake rates of pelagic ciliates at low bacterial concentrations refute both of these ideas. We and others have found that ciliates, including spirotrichous forms, present in the plankton of both coastal marine waters and lakes can be voracious consumers of bacteria^{5–8}. Using a new method for preparing monodisperse, fluorescently labelled 'marker' bacteria, we have measured bacterial clearance rates for pelagic ciliates which are 10–100 times greater than earlier estimates. In waters of a salt marsh estuary, ciliate grazing can account for 100% of estimated protozoan bacterivory. Ciliate bacterivory may 'short-circuit' the microbial loop in aquatic ecosystems by making bacterioplankton production available to metazoan grazers via a simple, two-step food chain.

Water samples containing natural assemblages of ciliates were collected in the Duplin River, a salt marsh tidal embayment on the Georgia, US coast. Ciliates were not taxonomically identified, except for one scuticociliate, *Uronema marina*, which we cultured on a wheat grain infusion. However, based on cell size, ciliature, and number and shape of cell nuclei, we were able to distinguish nine separate morphological types in our samples: four spirotrichs (class Spirotrichea, subclass Choreotrichia)⁹, four scuticociliates, including *U. marina* (class Oligohymenophorea, subclass Hymenostomatida)⁹, and one free-swimming, 30–40 μm peritrich (class Oligohymenophorea, subclass Peritrichia, order Mobilida)⁹. The spirotrichs and scuticociliates were generally <20 μm in size and numerically dominated the assemblage of pelagic ciliates, as previously noted for these coastal waters⁷.

Specific clearance rates based on uptake rates of fluorescently labelled marker bacteria (FLB) for the nine ciliate types, expressed as the number of body volumes cleared per cell per unit time, ranged from $4 \times 10^4 \text{ h}^{-1}$ to $6.8 \times 10^5 \text{ h}^{-1}$ (Fig. 1). These rates are 1–2 orders of magnitude higher than the previous specific clearance rates estimated for ciliates feeding on bacteria⁴. We also calculated, from data reported in two other recent studies in which fluorescent microspheres were used to evaluate ingestion of bacterial-sized particles by a marine *Cyclidium* sp (M. Pace and M. Bailiff, manuscript in preparation) and by two freshwater spirotrichs⁶, specific clearance rates 10–20-fold higher than the earlier estimates (Fig. 1). In several of our own experiments we included a separate treatment in which 0.5- μm

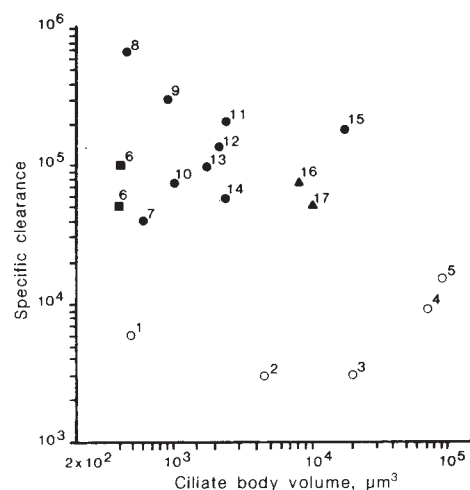


Fig. 1 Previous (open circles) and recent (solid symbols) cell-specific clearance rates (body volumes h^{-1}) determined for pelagic ciliates as a function of ciliate cell volume. 1, *Cyclidium glaucoma*; 2, *Glaucocoma scintillans*; 3, *Colpidium campylum*; 4, *Paramecium trichium*; 5, *P. caudatum* (○)⁴; 6, *Cyclidium* sp. (■) (M. Pace and M. Bailiff, manuscript in preparation); 7, *Uronema marina*; 8, Spirotrich a; 9, Spirotrich b; 10, Spirotrich c; 11, Spirotrich d; 12, Scuticociliate b; 13, Scuticociliate c; 14, Scuticociliate d; 15, Free-swimming peritrich (●; this study); 16, *Epistylis rotans*; 17, *Strombidium* sp. (▲; ref. 6—note that the specific clearance rates calculated by Borsheim were in error, too low by a factor of 10^3).

Methods. Experiments were carried out at 25 °C, approximately *in situ* temperature, in 400 ml Whirl-pak bags, presoaked in 10% HCl and copiously rinsed with deionized water. Short-term uptake of bacteria by ciliates present in the water was evaluated using preparations of a 0.6- μm (0.10 μm^3), gram-negative bacterium, isolated from local waters, which had been simultaneously heat-killed and stained with the fluoro-chrome 5-(4,6-dichlorotriazin-2-yl)-amino-fluorescein (DTAF) (B.F.S. *et al.*, manuscript in preparation). The fluorescently labelled bacteria (FLB) are not toxic to ciliates, and can be metabolized to support rapid ciliate growth (B. Sherr *et al.*, manuscript in preparation). FLB were added to samples of estuarine water at concentrations of $1-4 \times 10^5 \text{ ml}^{-1}$, which ranged from 3–5% of the natural bacterioplankton abundance, determined before initiating the experiment by acridine orange (AO) direct counting²⁴. After addition of FLB, 5 ml subsamples were removed from the bags and fixed with a final concentration of 2% borate-buffered formalin at time zero and at 5-min intervals for periods of 15–30 min. Time zero subsamples were examined for abundance of FLB by filtering 1.0 ml aliquots onto irgalan-black stained, 0.2 μm Nucleopore filters. A Zeiss Universal epifluorescence microscope outfitted with a 75-W xenon lamp was used for enumerating bacteria at 1250 $\times$, using an AO filter set (Zeiss 47 77 09); with this filter set the FLB fluoresced a bright apple-green. The average number of FLB ingested by each ciliate species with time was determined by filtering the time series subsamples onto 0.8- μm , black-stained Nucleopore filters, staining with the fluoro-chrome diamidinophenylindole (DAPI)²⁵, and examining the prepared filters by epifluorescence microscopy. Ciliates were found on the filter at a low magnification ($\times 160$) using a DAPI filter set (Zeiss 47 77 02); then the number of FLB within an individual ciliate was counted under oil immersion at $\times 1,250$ using the AO filter set. The rate of uptake of FLB by ciliates was generally linear for 10–20 min, after which the average number of FLB per cell levelled off as digestion of the bacteria approximated the rate of ingestion. We do not know whether the ciliates might have preferentially ingested FLB; however, addition of only trace amounts of FLB to the total population of suspended bacteria probably minimized this potential problem.

fluorescent latex microspheres (Polysciences, Inc.) were added at approximately the same concentration as the FLB (B.F.S. *et al.*, manuscript in preparation). We found that the scuticociliates and the peritrich species ingested the microspheres and FLB at equivalent rates; however, the spirotrichs consistently ingested the FLB at a rate 5-fold higher than for the microspheres.

Table 1 Rate of ciliate consumption of bacteria

Date	Bacterioplankton abundance at T_0 (10^6 cells ml^{-1})	Bacterivory (10^5 cells consumed $\text{ml}^{-1} \text{h}^{-1}$)	
		Total consumption	Ciliate consumption
7 June 1985	8.4	1.7	1.9
1 July 1985	6.9	1.8	2.4
16 September 1985	5.5	0.6	1.2
23 September 1985	3.8	0.3	0.2

Comparison of estimates of total bacterivory by $<17\text{-}\mu\text{m}$ estuarine protozoa using prokaryotic inhibitors to stop bacterial growth¹⁷, with estimates of ciliate bacterivory calculated from the population abundance of $<17\text{-}\mu\text{m}$ ciliates determined for each experiment and the ciliate clearance rates measured here.

The highest clearance rate in our study was found for a $8\text{--}10\text{-}\mu\text{m}$ diameter spirotrich (Fig. 1). We calculated that if this ciliate fed continuously at such a rate, it would be able to ingest sufficient biomass to sustain one doubling per day, assuming a 33% gross growth efficiency, at a bacterioplankton concentration of $15\text{ }\mu\text{g}$ carbon per litre. In our estuarine waters, where the average bacterial cell size is about $0.08\text{ }\mu\text{m}^3$ (ref. 10), this biomass would equal 8.5×10^5 cells ml^{-1} , using a conversion factor of $0.22\text{ pgC }\mu\text{m}^{-3}$ bacterial biovolume¹¹. The idea that pelagic ciliates can grow at the expense of such a low bacterial abundance is supported by the finding of Rivier *et al.*⁸ that a $20\text{--}30\text{-}\mu\text{m}$ spirotrich, *Strombidium sulcatum*, isolated from oligotrophic waters of the northwestern Mediterranean, grew in laboratory cultures on $3\text{--}6 \times 10^5$ bacteria per ml. The other ciliates examined in the present study (Fig. 1) would require a higher density of bacteria to sustain one doubling per day, up to $170\text{ }\mu\text{gC l}^{-1}$, or 9.6×10^6 cells ml^{-1} . This range of bacterial abundance, $15\text{--}170\text{ }\mu\text{gC l}^{-1}$, is typical of coastal marine waters^{12,13}, and also of freshwater systems^{14–16}.

We have previously determined rates of bacterivory by phagotrophic protozoa in $17\text{-}\mu\text{m}$ -screened estuarine water using a selective inhibitor approach¹⁷. Based on the clearance rates found in the present study, we calculated the potential contribution of the $<17\text{-}\mu\text{m}$ ciliates counted in prokaryotic-inhibited samples to the total protozoan bacterivory determined for four summer experiments. We used an average per cell clearance rate (in this case volume of water cleared $\text{cell}^{-1} \text{h}^{-1}$) of 140 nl h^{-1} for scuticociliates and 270 nl h^{-1} for spirotrichs. Comparison of the rates of bacterial loss determined in inhibited treatments with the calculated rates of ciliate bacterivory (Table 1) shows that ciliate grazing could account for virtually all of the decrease in bacteria observed in each experiment.

The FLB method we developed to study instantaneous protozoan bacterivory *in situ* is a significant advance over the use of fluorescent microspheres⁶, as bacterivorous spirotrichs apparently discriminate against inert particles. Our results indicate that, at least in estuarine waters, there can be a quantitatively significant trophic pathway between bacteria and ciliates in the pelagic food web. This has important implications for the present controversy regarding the contribution of microbial production to metazoan food chains. Ciliates are readily consumed by metazooplankton, including micro-crustaceans and fish larvae^{18,19}. Therefore ciliate bacterivory can by-pass at least one step in the bacteria to flagellate to ciliate microbial loop²⁰, which is currently believed to be a sink rather than a link for carbon flow in pelagic ecosystems^{21,22}, and make a substantial fraction of the often considerable biomass production of bacteria available to higher order consumers in the food web. As pelagic ciliates are abundant in many parts of the world ocean^{7,12}, as well as in lakes²³, their role as bacterial grazers is in urgent need of reevaluation.

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1. Sieburth, J. M. in *Heterotrophic Activity in the Sea* (eds Hobbie, J. E. & Williams, P. J. leB.) 405–444 (Plenum, New York, 1984).
2. Fenchel, T. in *Flows of Energy and Materials in Marine Ecosystems* (ed. Fasham, M. J. R.) 301–316 (Plenum, New York, 1984).
3. Fenchel, T. *Microb. Ecol.* **6**, 1–11 (1980).
4. Fenchel, T. *Microb. Ecol.* **6**, 13–25 (1980).
5. Gast, V. *Mar. Ecol. Prog. Ser.* **22**, 107–120 (1985).
6. Borsheim, K. Y. *Oecologia* **63**, 286–288 (1984).
7. Sherr, E. B., Sherr, B. F., Fallon, R. D. & Newell, S. Y. *Limnol. Oceanogr.* **31**, 177–183 (1986).
8. Rivier, A., Brownlee, D. C., Sheldon, R. W. & Rassoulzadegan, F. *Mar. Microb. Food Webs* **1**, 51–60 (1986).
9. Lee, J. J., Hutner, S. Y. & Bovee, E. C. (eds) *An Illustrated Guide to the Protozoa* (Allen, Lawrence, Kansas, 1985).
10. Newell, S. Y. & Christian, R. R. *Appl. envir. Microbiol.* **42**, 23–31 (1981).
11. Bratbak, G. & Dundas, I. *Appl. microb. Microbiol.* **48**, 755–757 (1984).
12. Sorokin, Y. I. in *Analysis of Marine Ecosystems* (ed. Longhurst, A. R.) 293–342 (Academic, London, 1981).
13. Van Es, F. B. & Meyer-Reil, L.-A. *Adv. microb. Ecol.* **6**, 111–170 (1982).
14. Hobbie, J. E. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* **12**, 59–63 (1979).
15. Pedros-Alio, C. & Brock, T. D. *Appl. environ. Microbiol.* **44**, 203–218 (1982).
16. Riemann, B., Fuhrman, J. & Azam, F. *Microb. Ecol.* **8**, 101–114 (1982).
17. Sherr, B. F., Sherr, E. B., Andrew, T. L., Fallon, R. D. & Newell, S. Y. *Mar. Ecol. Prog. Ser.* **32**, 169–180 (1986).
18. Porter, K. G., Pace, M. L. & Battey, J. F. *Nature* **277**, 563–565 (1979).
19. Sherr, E. B., Sherr, B. F. & Paffenhofer, G.-A. *Mar. Microb. Food Webs* **1**, 61–80 (1986).
20. Azam, F. *et al. Mar. Ecol. Prog. Ser.* **10**, 257–263 (1983).
21. Williams, P. J. leB. in *Flows of Energy and Materials in Marine Ecosystems* (ed. Fasham, M. J. R.) 271–300 (Plenum, New York, 1984).
22. Ducklow, H. W., Purdie, D. A., Williams, P. J. leB. & Davis, J. M. *Science* **232**, 865–867 (1986).
23. Pace, M. *Can. J. Fish. aquat. Sci.* **39**, 1106–1116 (1982).
24. Hobbie, J. E., Daley, R. J. & Jasper, S. *Appl. environ. Microbiol.* **33**, 1225–1228 (1977).
25. Porter, K. G. & Feig, Y. S. *Limnol. Oceanogr.* **25**, 943–948 (1980).

Regeneration by supernumerary axons with synaptic terminals in spinal motoneurons of cats

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Axons in the central nervous system (CNS) of mammals do not normally regrow if they are cut, which severely limits restoration of function after injury. We have studied the reactions of adult cat spinal α -motoneurons after chronic transection of their axons in the periphery by labelling single cells with horseradish peroxidase. Twelve weeks after the operation, about a third of the axotomized cells had developed a 'supernumerary' axon originating from the cell-body region. These supernumerary axons had variable trajectories and termination fields in the ipsilateral spinal cord but generally anomalous projections. Ultrastructural examination shows that they give rise to boutons that form morphologically normal synaptic contacts with neuronal profiles, although they contain dense-cored vesicles not normally seen^{1,2} in central terminals of α -motor axons. We conclude that axotomized neurons in the mammalian CNS may be able to form new synaptic contacts by means of supernumerary axons in the absence of local damage.

The medial gastrocnemius (MG) nerve of adult cats was transected in the left popliteal fossa. The central stump was ligated and prevented from reinnervating the muscle. Twelve weeks later, glass micropipettes filled with a 25% solution of horseradish peroxidase (HRP; Sigma, type VI) in 0.1 M NaOH and 0.5 M KCl were used for iontophoretic injection of HRP intracellularly into single chronically axotomized MG α -motoneurons^{3–8}. The motoneurons were identified by the anti-

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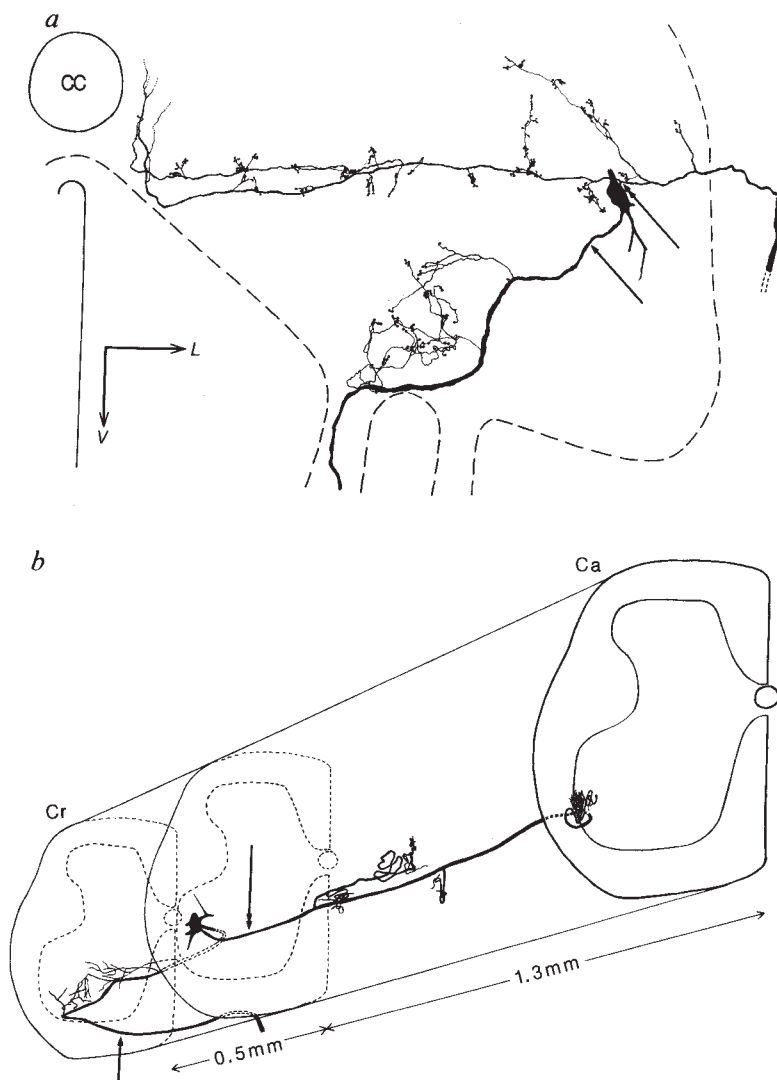


Fig. 1 *a*, Reconstruction in the transverse plane of the axonal systems of a chronically axotomized MG α -motoneuron after intracellular labelling with HRP. The original stem motor axon (single arrow) and the supernumerary axon (double arrow) had separate origins from the cell soma (see Fig. 2). The original stem axon exhibited a conventional ventromedial course and left the spinal cord by the ventral root. It gave rise to three axon collateral systems in the grey matter. The supernumerary axon divided early into two main branches, which elongated in opposite directions. The medial branch extended towards the central canal (CC) and gave rise to multiple thin collateral branches, which terminated in clusters of small bouton-like swellings. The lateral branch entered the lateral funiculus, where it ascended beyond the end of the section series (>920 μ m rostrally to the cell soma). It produced two collateral branches within the grey matter. Broken line, border between the spinal grey and white matter; L, lateral direction; V, ventral. *b*, Schematic reconstruction of a portion of the lumbosacral spinal cord together with the intramedullary axonal systems of a chronically axotomized MG α -motoneuron after intracellular labelling with HRP. The original stem motor axon (single arrow) and the supernumerary axon (double arrow) had a common origin from the cell body (see Fig. 3). The original axon gave rise to four axon collateral systems in the grey matter and left the spinal cord through the ventral root. The supernumerary axon descended in the ventrolateral grey matter and terminated 1.3 mm caudally to the cell body. It exhibited few collateral branches, all of unusually large diameter. The collaterals formed tangles and possessed few bouton-like specializations. Ca, caudal; Cr, cranial.

dromic action potentials evoked by stimulation of the central stump of the cut MG nerve. After glutaraldehyde fixation, serial sectioning and histological processing of the spinal cord tissue³⁻⁸, light microscopic reconstructions and ultrastructural analyses of the HRP-labelled axotomized MG α -motoneurons were performed.

Twenty-six chronically axotomized MG α -motoneurons in six cats were studied. In each, the original stem motoraxon still remained (Fig. 1 *a* and *b*, single arrows) and generally gave off one or more axon collateral systems in the ventral grey matter before leaving the spinal cord through the L7 or S1 ventral root. The intramedullary trajectories and projection areas of these original axons and axon collateral systems were broadly similar to those observed in normal non-axotomized α -motoneurons¹⁻⁵.

In addition to the original stem motoraxon, eight of the axotomized α -motoneurons in four cats were found to possess an intramedullary supernumerary axon (Fig. 1 *a* and *b*, double arrows). Such supernumerary axons have previously not been observed in extensive studies on normal HRP-stained triceps surae³⁻⁹ or phrenic^{10,11} α -motoneurons of adult cats. We therefore concluded that the appearance of supernumerary axons in the present axotomized neurons was related to the chronic nerve injury.

The supernumerary axons originated from the cell-body region of the axotomized cells (Figs 1, 2 and 3) and usually exhibited a fairly uniform diameter along their length; no more than one supernumerary axon was detected per axotomized MG

α -motoneuron. All supernumerary axons were myelinated (Figs 2 and 3), but the degree of myelination varied from the normal appearance with regular nodes of Ranvier to a more patchy and irregular distribution of myelin along the axon. All the supernumerary axons gave off collateral branches, which generally formed bouton-like specializations of both *en passant* and terminal types (Fig. 1 *a*). The supernumerary axons demonstrated a considerable variation in their trajectories and areas of termination. Compared with the situation in normal animals¹⁻⁵ the projections were generally anomalous. In this material, the supernumerary axons projected ventrolaterally within Rexed's¹² lamina IX, ventromedially into laminae VII and VIII, medially towards the central canal (Fig. 1 *a*) or dorsally into laminae I-IV. In some cases they passed within the ventral or lateral funiculi for varying distances (Fig. 1 *a*, *b*). However, they were never seen to extend into the ventral or dorsal roots or to leave the spinal cord elsewhere, nor did they cross over to the contralateral side of the spinal cord.

The ultrastructure of 14 bouton-like specializations originating from the supernumerary axons of two axotomized MG α -motoneurons was examined with the electron microscope. All these specializations were found to contact neuronal profiles and they contained mitochondria as well as numerous dense-core, spheroid vesicles of 35-65 nm diameter (Fig. 4). The latter observation was unexpected, because intramedullary boutons of normal triceps surae motoraxon collaterals contain only clear, spheroid synaptic vesicles^{1,2}. In most of the supernumerary

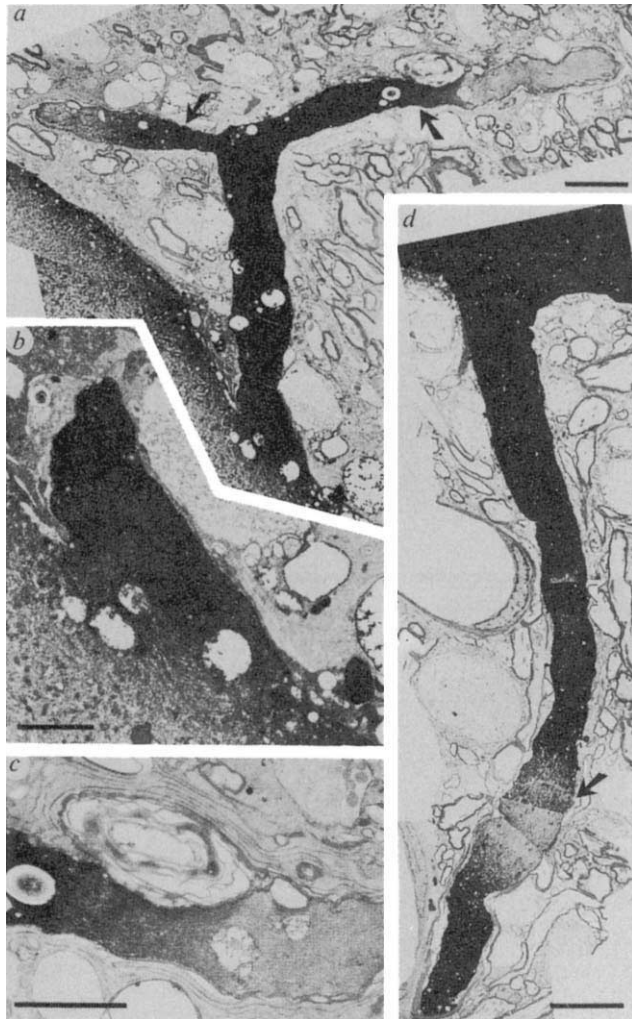


Fig. 2 Electron micrographs of the intracellularly HRP-labelled and chronically axotomized MG α -motoneuron illustrated in Fig. 1a. *a*, Photomontage showing the supernumerary axon originating from the cell body and dividing into a lateral (right) and medial (left) branch. Arrows, beginnings of the thin myelin sheaths surrounding the two axonal branches; calibration bar, 5 μ m. *b*, The cell-body origin of the supernumerary axon shown at higher magnification; calibration bar, 2.5 μ m. *c*, Start of the myelin sheath surrounding the lateral branch of the supernumerary axon shown at higher magnification; calibration bar, 3 μ m. *d*, Photomontage showing the original stem motor axon leaving the cell body (top) at another level of the section series. Arrow, beginning of the myelin sheath; calibration bar, 8 μ m.

Fig. 3 Electron micrographs of the intracellularly HRP-labelled and chronically axotomized MG α -motoneuron illustrated in Fig. 1b. *a*, Photomontage showing the common starting point of the original stem motor axon (single arrow) and the supernumerary axon (double arrow) from the cell body. The supernumerary axon had a straight caudal direction from its origin, which explains its lack of extension in the transverse section plane. Calibration bar, 15 μ m. *b*, The supernumerary axon located in the lateral white column of the spinal cord about 1.2 mm caudally to the level of the parent cell body. The axon is myelinated and contains HRP reaction product. Calibration bar, 3 μ m.

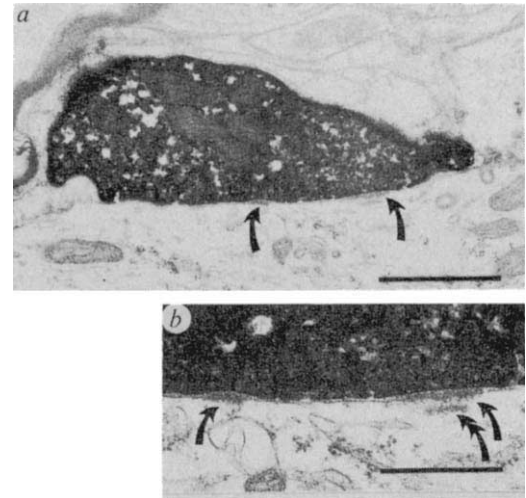
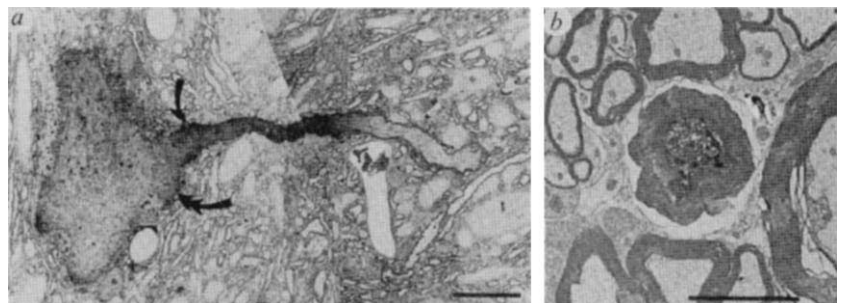


Fig. 4 *a*, Electron micrograph of an HRP-labelled bouton originating from the supernumerary axonal system illustrated in Fig. 1a. The bouton exhibits mitochondria, many dense-cored vesicles and a neuronal target of apposition. Arrows, synaptic contact zones; calibration bar, 1 μ m. *b*, Synaptic contact zones shown at higher magnification. The synaptic clefts contain dense material and there is also dense cytoplasmic material located at the postsynaptic membrane (arrows). A ribbon of subjunctional dense material (double arrow) is associated with the right contact zone. Calibration bar, 0.5 μ m.

boutons the synaptic character was further emphasized by the presence of synaptic clefts with postsynaptic dense material (Fig. 4). Presynaptic densities would not be identified owing to the presence of the intracellular HRP reaction product.

Regenerative elongation of axons or axon-like structures in the central nervous system of mammals has previously been demonstrated in association with central injuries involving the formation of glial or mesodermal scar tissue^{13,14}. However, the myelinated processes of uniform diameter that originate from distal dendritic branches after intramedullary axotomy of cat α -motoneurons¹⁴ do not exhibit intramedullary collaterals or bouton-like structures. Furthermore, only dendrites in close proximity to the site of spinal injury were seen to generate such 'dendraxons', all of which elongated in the direction of the scar tissue, so the possibility remains that only lesioned dendrites may have developed the 'dendraxons' or that some trophic influence of the scar tissue may have triggered the 'axonalization' of adjacent dendrites.

Several investigators have also demonstrated elongation of central axons into peripheral nerve tissue grafted into the mammalian brain or spinal cord¹⁵⁻²⁰. This extension or sprouting of central axons is assumed to be associated with trophic factors within the grafted peripheral nerve tissue. The present morphological study demonstrates that regenerative axonal growth within the adult mammalian central nervous system may involve the formation of supernumerary axons originating from the cell body region, and that this regenerative growth can take place in the absence of local changes in the neuroglial environment

caused by scar tissue or peripheral nerve grafts. Furthermore, the supernumerary axons are capable of generating boutons, which according to ultrastructural criteria form regular synaptic contacts with neuronal structures. A previously undescribed form of regenerative synaptogenesis in the central nervous system is therefore suggested.

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1. Lagerbäck, P.-Å., Ronnevi, L.-O., Cullheim, S. & Kellerth, J.-O. *Brain Res.* **207**, 247-266 (1981).
2. Lagerbäck, P.-Å., Ronnevi, L.-O., Cullheim, S. & Kellerth, J.-O. *Brain Res.* **222**, 29-41 (1981).
3. Cullheim, S. & Kellerth, J.-O. *J. comp. Neurol.* **178**, 537-558 (1978).
4. Cullheim, S. & Kellerth, J.-O. *J. Physiol., Lond.* **281**, 285-299 (1978).
5. Cullheim, S. & Kellerth, J.-O. *J. Physiol., Lond.* **281**, 301-313 (1978).
6. Ulfhake, B. & Kellerth, J.-O. *J. comp. Neurol.* **202**, 571-583 (1981).
7. Ulfhake, B. & Kellerth, J.-O. *Brain Res.* **251**, 201-209 (1982).
8. Ulfhake, B. & Kellerth, J.-O. *Brain Res.* **264**, 1-19 (1983).
9. Burke, R. E. *et al. J. comp. Neurol.* **209**, 17-28 (1982).
10. Cameron, W. E., Averill, D. B. & Berger, A. J. *J. comp. Neurol.* **219**, 70-80 (1983).

11. Lipski, J., Fyffe, R. E. W. & Jodkowski, J. J. *Neurosci.* **5**, 1545-1555 (1985).
12. Rexed, B. J. *comp. Neurol.* **100**, 297-351 (1954).
13. Risling, M., Cullheim, S. & Hildebrand, C. *Brain Res.* **280**, 15-23 (1983).
14. Lindå, H., Risling, M. & Cullheim, S. *Brain Res.* **358**, 329-333 (1985).
15. Kao, C. C., Chang, L. W. & Bloodworth Jr, J. M. B. *Expl. Neurol.* **54**, 591-615 (1977).
16. Richardson, P. M., McGuinness, U. M. & Aguayo, A. J. *Nature* **284**, 264-265 (1980).
17. David, S. & Aguayo, A. J. *Science* **214**, 931-933 (1981).
18. Benfey, M. & Aguayo, A. J. *Nature* **296**, 150-152 (1982).
19. Wrathall, J. R., Rigamonti, D. D., Braford, M. R. & Kao, C. C. *Acta Neuropathol.* **57**, 59-69 (1982).
20. Sceats Jr, D. J., Friedman, W. A., Syper, G. W. & Ballinger Jr, W. E. *Brain Res.* **362**, 149-156 (1986).

Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons

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The modulation of voltage-activated calcium currents by protein kinases provides excitable cells with a mechanism for regulating their electrical behaviour¹⁻⁷. At the single channel level, modulation of calcium current has, to date, been characterized only in cardiac muscle, where β -adrenergic agonists, acting through cyclic AMP-dependent protein kinase, enhance the calcium current by increasing channel availability and opening^{3,8-12}. We now report that enhancement of calcium current in the peptidergic bag cell neurons of *Aplysia* by protein kinase C (ref. 13) occurs through a different mechanism, the recruitment of a previously covert class of calcium channel. Under control conditions, bag cell neurons contain only one class of voltage-activated calcium channel with a conductance of ~ 12 pS. After exposure to agents that activate protein kinase C, these neurons also express a second class of calcium channel with a different unitary conductance (~ 24 pS) that is never seen in untreated cells.

The function of the neurosecretory bag cell neurons is to initiate a sequence of reproductive behaviours. Activation of these cells leads to major changes in their electrical properties, controlled in part by activation of protein kinase C⁷. This calcium-, phospholipid-, diacylglycerol-dependent enzyme is present in high concentrations in the nervous system of *Aplysia*¹⁴ as well as in mammalian brain¹⁵. In intact cells, the enzyme may be activated by phorbol esters such as TPA (12-O-tetradecanoyl-phorbol-13-acetate; PMA) or synthetic diacylglycerols such as DOG (1, 2-dioctanoyl glycerol). In isolated bag cell neurons, injection of purified protein kinase C, or application of TPA or synthetic diacylglycerols enhances evoked action potentials¹³. In whole-cell voltage clamp experiments, TPA pretreatment increases the magnitude of the calcium current although voltage-activated potassium currents are unaffected¹³.

Table 1 Unitary Ca channel activity in the absence and presence of activators of protein kinase C

	No. of patches	No channels (%)	Small channels (%)	Large channels (%)
Control	33	64	36	0
TPA pretreatment (10-20 nM)	28	43	36	36
TPA pretreatment (100-200 nM)	6	0	67	50
DOG pretreatment (1.5 $\mu\text{g ml}^{-1}$)	3	0	33	66
TPA applied after patch formation	11	55	45	0
TPA in pipette	8	50	50	0

Recording conditions as described in Fig. 1 legend unless otherwise noted. Inward channels were classified as small (slope conductance < 16 pS) or large (> 20 pS). Cells were pretreated with TPA for 10-120 min, or with DOG for 14-40 min. Shorter pretreatments were not attempted because enhancement of spike height by TPA requires 2-5 min in cells impaled with microelectrodes¹³. In experiments in which TPA was included in the recording pipette or added to the bath after formation of the seal, patches were monitored for 10-60 min with no apparent large channel activity. The apparent increase in the number of small channels by very high doses of TPA (line 3) was not statistically significant (Fisher's exact test, $P = 0.10$). We did not attempt many experiments under these conditions because such doses of TPA are sometimes seen to induce spontaneous activity in cells impaled with microelectrodes.

We used patch clamp recording techniques¹⁶ to study the mechanism of the enhanced Ca current at the single channel level. Cell-attached patch recordings were made on bag cell neurons maintained in primary culture, using pipettes containing 185 mM Ba^{2+} rather than Ca^{2+} as the charge carrier to facilitate resolution of unitary calcium channel currents⁸. In normally polarized cells, depolarization of the membrane patch 20-30 mV above the resting potential evoked inward unitary current events such as those shown in Fig. 1a. This type of activity was characterized by a slope conductance of < 16 pS, and was routinely observed in both control patches and patches on cells pretreated with 10 nM TPA (Table 1). Cells pretreated with TPA expressed an additional kind of channel activity, distinguished by a larger unitary amplitude at any given potential, and a slope conductance of ≥ 20 pS. The large conductance channel was also induced when protein kinase C was stimulated by pretreatment of

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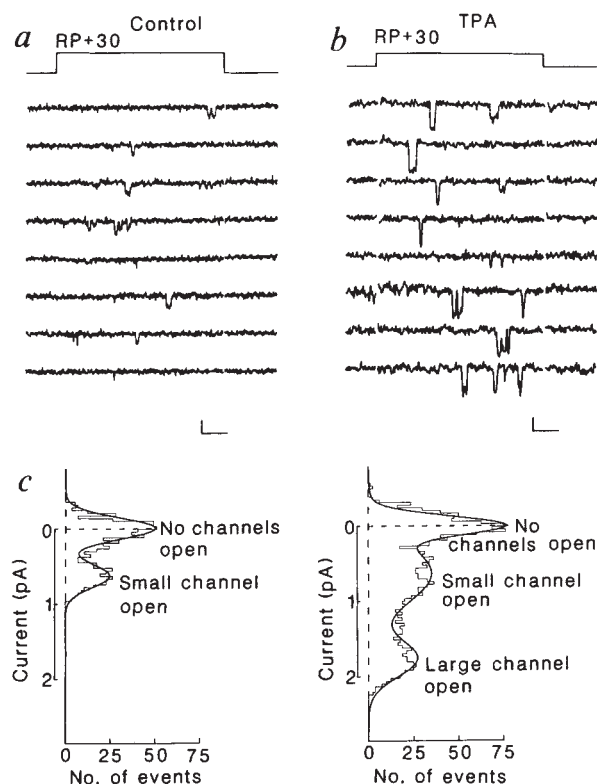


Fig. 1 Examples of two types of inward channel activity in cell-attached patches on bag cell neurons in primary culture. Downward deflections correspond to inward current. Leak and capacitive currents have been subtracted. Scale bars (vertical) 1 pA, (horizontal) 20 ms in *a*, 10 ms in *b*. *a*, A single class of inward current events observed during depolarizations from (*RP* - 20) to (*RP* + 30) mV, where *RP*, the resting potential was estimated as ~ -48 mV (Fig. 2 legend). *b*, Inward current events of two distinct sizes in another cell pretreated with 10 nM TPA. Pulses from *RP* to (*RP* + 30) mV. Estimated *RP* ~ -61 mV. *c*, Histograms of current amplitude for the experiments shown in *a* (left) and *b* (right). The left histogram is fit with the sum of two gaussian functions about 0 and 0.72 pA; the right with the sum of three gaussian functions about 0, 0.69 and 1.74 pA.

Methods. *Aplysia* bag cell neurons were enzymatically and mechanically isolated, plated onto plastic tissue culture dishes, and maintained in primary culture for 1–5 days²⁶. Cells were incubated in modified Eagle's medium (MEM) containing 460 mM NaCl, 10.4 mM KCl, 27.5 mM MgCl₂, 27.5 mM MgSO₄, 11 mM CaCl₂, 5.5 mM glucose, and 15 mM HEPES-NaOH (pH 7.8), supplemented with essential and nonessential amino acids, vitamins, penicillin and streptomycin (Grand Island Biological). During recordings, cells were bathed in medium containing chloride in place of sulphate and, in some cases, with supplements omitted. Single channel recordings were made in the cell-attached configuration¹⁶, with Sylgard-coated patch pipettes containing 185 mM BaCl₂, 15 mM HEPES-TEA-OH (pH 7.8). TEA-Cl (260 mM), CsCl (10 mM), 4-aminopyridine (20 mM) were also included in the pipette solution to help block outward channels; preliminary experiments showed that outward channel activity was often detectable when the pipette contained isotonic BaCl₂ only. Phorbol ester stock solutions were 1–5 mM in DMSO, and were stored at -5°C . This and all subsequent current records were filtered with a corner frequency of 1 kHz and sampled at 5 kHz, except the record in Fig. 1*b* which was sampled at 10 kHz (note change in timescale).

neurons with the synthetic diacylglycerol DOG (Figs 2 and 3; Table 1). Some patches contained both a large and small channel. An example can be seen in Fig. 1*b*, where openings of two different sizes can be seen (1st and 3rd traces), and the histogram of current amplitudes has two non-zero peaks (Fig. 1*c*).

Further evidence for the existence of two distinct channel sizes is shown in Fig. 2. Figure 2*a* shows results from 10 patches

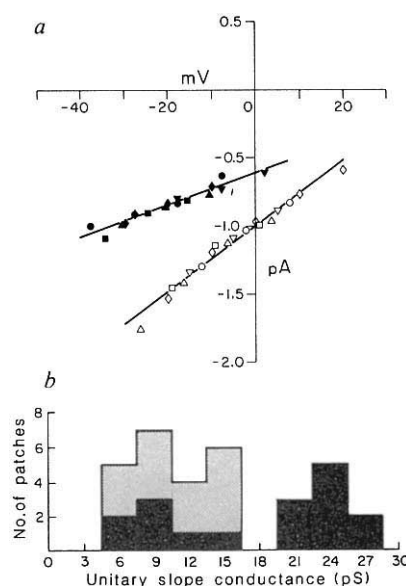


Fig. 2 *a*, Unitary current amplitudes as a function of potential for control channels and channels induced by stimulation of protein kinase C. In each of 10 different patches, averaged measurements of the magnitude of single channel currents were made at 3 or more potentials. The conductance of each channel (γ) was determined by linear regression. Solid symbols, open channel currents of 'small' channels (defined as those with $\gamma < 16$ pS; average value, 11.6; range 8.7–13.8), measured in 5 different patches ($\blacklozenge, \blacktriangledown$, control cells; $\bullet$, TPA-pretreated cell; $\blacktriangle, \blacksquare$, recordings with pipettes containing TPA). The points have been fitted with a straight line with a slope conductance of 11.8 pS and a voltage intercept of 52 mV. Open symbols, open channel currents of 'large' channels (defined as those with $\gamma > 20$ pS; average value, 23.8; range 22.7–27.8), measured in 5 different patches ($\square, \diamond, \triangle$, cells pretreated with TPA; $\nabla, \circ$, cells pretreated with DOG). Points are fitted with a straight line with a slope of 24.0 pS and voltage intercept of 42 mV. Measurements from one large and one small channel ($\blacklozenge, \diamond$) are from cells depolarized to 0 mV by an extracellular medium containing: potassium aspartate (535 mM), potassium EGTA (15 mM) and HEPES-KOH (15 mM, pH 7.8). These two channels were used to estimate resting potentials in other patch clamp recordings. Resting potentials estimated in this way ranged from -20 to -60 mV; a very wide range of resting potentials is also observed with microelectrode recordings. *b*, Distribution of slope conductances for channels included in Table 1. Stippled area, channels from control cells and from recordings made with TPA inside the pipette; solid area, channels from cells pretreated with TPA (10–200 nM) or DOG (1.5 $\mu\text{g ml}^{-1}$). Because *b* includes estimates of single channel conductances based on measurements at only two potentials it has greater scatter in conductance values than *a*. No data from patches containing more than one size of channel are included.

in which the single-channel current was accurately measured at three or more different potentials. The single channel conductances determined by linear regression clustered about two values, 12 and 24 pS, and none of the 10 channels had conductance values between 14 and 22 pS. The large channels were seen only in cells pretreated with activators of protein kinase C. As Fig. 2*a* shows, the conductance of the small channel is the same in control and TPA-treated cells. The slope conductance of the large channel is the same in TPA-pretreated and DOG-pretreated cells. For both small and large channels, the current-voltage relation extrapolates to a positive reversal potential. The true reversal potential is likely to be even more positive, if these channels show the marked rectification characteristic of calcium channels studied in other systems^{17–20}.

The results illustrated in Figs 1 and 2*a* are representative of a large number of cell-attached patches (Table 1, Fig. 2*b*). Large

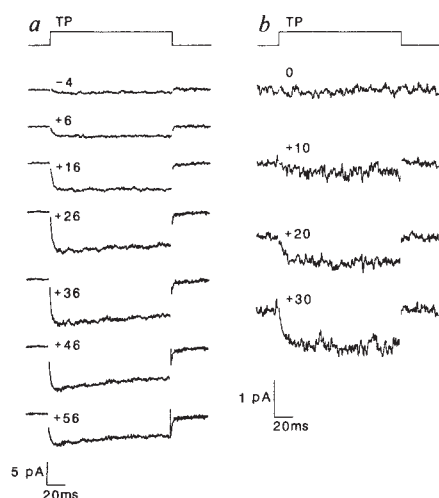


Fig. 3 Voltage dependence and time dependence of currents associated with activity of small and large conductance channels. *a*, Mean currents obtained from a patch containing many small conductance channels and no detectable activity of large conductance channels. Estimated holding potential -54 mV, test potentials (TP) as indicated. No drug pretreatment. At $+30$ mV, the ratio of peak current to single channel current is 25. This represents a lower bound on the number of channels in the patch, as the probability of a channel being open is much less than 1. *b*, Mean currents obtained from a patch containing at least 5 large conductance channels and no detectable activity of small conductance channels. Holding potential -20 mV, test potentials as indicated. Pretreatment with $1.5 \mu\text{g ml}^{-1}$ DOG.

conductance channels were never seen in 34 control patches (untreated cells, $n=21$; cells pretreated with the inactive analogue 4- α -phorbol, $n=13$). They were found in 36% of all patches from cells pretreated with low concentrations of TPA (10–20 nM).

In Fig. 3, the voltage dependence and time dependence of the small and large conductance Ca channels are compared. Figure 3*a* shows signal-averaged current traces from a control patch with at least 25 small conductance channels; Fig. 3*b* shows records from a patch on a DOG-pretreated cell with about 5 large conductance channels but no detectable activity of the other type. Both channel types become active in a steeply voltage-dependent manner over the range of potentials from 0 to $+30$ mV. In both cases, the average current activates rapidly and shows little inactivation over the course of a 136 ms depolarizing pulse. In all these respects, the behaviour of the two types of channels is consistent with the pattern of modulation found previously in whole-cell recordings where TPA pretreatment enhanced the magnitude of the calcium current without obvious changes in the voltage or time dependence of the current¹³.

A detailed analysis of unitary recordings was carried out to compare the open-closed kinetics of the two types of channels. Figure 4 shows results from patches with only the small conductance channel (left) or only the large conductance channel (right). There is no striking difference in the kinetics of the small and large conductance channels. In both cases, the open time distribution is fitted by a single exponential with a time constant <1 ms, and the cumulative distribution of the latency to first opening rises relatively slowly. Openings of the large conductance channels showed a greater tendency to appear grouped in closely spaced bursts (see Fig. 4*e*).

In addition to differences in single channel conductance and bursting kinetics, another distinguishing characteristic may be spatial distribution. In 25% of the patches containing small channels we observed clusters of dozens of channels (Fig. 3*a*); this was never true for the large conductance channels. In hope

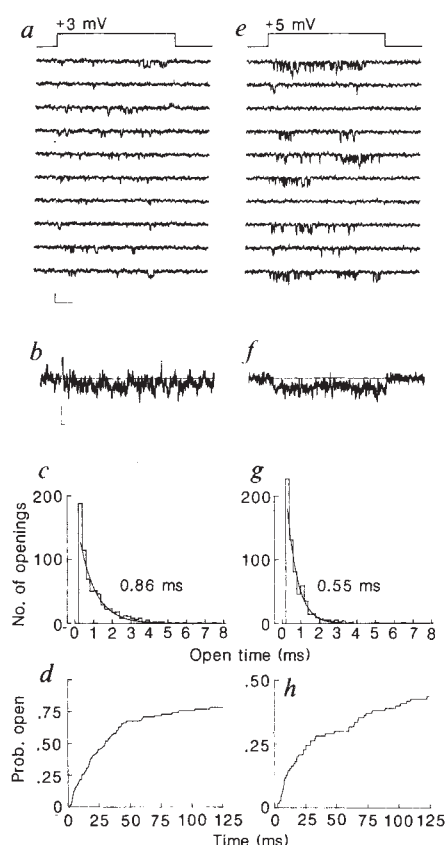


Fig. 4 Kinetics of small and large conductance channel openings studied in cell-attached patch recordings. Recording conditions as in Fig. 1. *a–d*, Analysis of small conductance channel openings associated with patch depolarization from -47 to $+3$ mV in a two-channel patch. No drug pretreatment. *e–h*, Activity of large conductance channel produced by patch depolarization from -95 to $+5$ mV in a two-channel patch. Pretreatment with 10 nM TPA. *a, e*, Sets of 10 consecutive records. Scale bars 1 pA and 20 ms (*a, b, e, f*). *b, f*, Associated mean current records, obtained by averaging more than 100 current traces. *c, g*, Distributions of open times; smooth curve indicates least-squares single exponential fit. *d, h*, Cumulative distribution of latency-to-first-opening (corrected for the presence of two channels). Similar values were obtained in analysis of one other small channel patch and three other large channel patches. Openings were defined as events in which the current exceeded half of the unit current size for at least 2 sample points. Note that vertical scales are different in *d* and *h*.

of finding clear-cut pharmacological differences between the two types of calcium channel, we tested a variety of calcium channel blockers. La^{3+} (5–30 μM) and nifedipine (5–10 μM) blocked calcium currents with similar effectiveness in whole cell recordings from control and TPA-pretreated cells. No clear results were obtained with Cd (2–50 μM) which appeared to be toxic to the cells.

These experiments demonstrate that modulation of Ca entry in bag cell neurons occurs through the recruitment of a covert class of Ca channels that are readily distinguished from the Ca channels that are active in unstimulated cells. Although we cannot completely rule out the possibility that the large channels arise from conversion of small channels, several pieces of evidence argue against this hypothesis. The percentage of patches containing small channels does not decrease in TPA-treated cells (Table 1), and, moreover, the two channels differ not only in single channel conductance and bursting kinetics but also in their spatial distribution.

The mechanism of Ca channel regulation that we have

described seems fundamentally different from the pattern of modulation found so far in other systems of voltage-gated ion channels. Previous studies have described modulation of ion current arising from changes in the availability or opening probability of an apparently homogeneous class of channels. This is true for L-type Ca channels in heart muscle cells⁸⁻¹² or S-type K channels in *Aplysia* sensory neurons²¹, two particularly well-studied cases where channels are modulated through the action of cAMP-dependent protein kinase.

Several pieces of evidence favour the idea that the large conductance Ca channel appears as a result of activation of protein kinase C. (1) In the experiments with TPA, the concentration of the phorbol ester (10 nM) is similar to that needed to activate protein kinase C in homogenates of *Aplysia* nervous system^{13,14}. (2) In mammalian brain, protein kinase C is the only known receptor of phorbol esters at such concentrations²². (3) No large channels were seen in cells treated with 4- α -phorbol, a phorbol compound that does not activate protein kinase C. (4) Large channels were also induced by pretreatment of bag cell neurons with DOG, a diacylglycerol activator of protein kinase C. The appearance of the large conductance channels cannot be secondary to changes in electrical activity as microelectrode recordings indicate that 10–20 nM TPA has no effect on resting potential and does not initiate spontaneous activity¹³.

An intriguing aspect of the induction of the large conductance channel is that the process is somehow prevented by the formation of cell-attached patches (Table 1). Although large channels were found in 63% of the non-blank patches on cells pretreated with TPA (10 nM), they were never found when TPA (100 nM) was added to the bath after formation of the patch (6 patches with no channel, 5 patches with small conductance channel). In addition, no large channels were observed in 8 recordings made with 10 nM TPA in the patch pipette solution. In this vein it is worth noting that the acute effect of TPA is also prevented by intracellular dialysis of bag cell neurons during whole cell recording (although TPA enhancement of Ca action potentials is readily observed with conventional microelectrodes). The recruitment of large conductance Ca channels may depend upon cellular constituents or structural elements of the native cytoplasm that are lost during dialysis or disrupted by formation of cell-attached patches.

The results in this paper provide a possible mechanism for effects of protein kinase C stimulation in other neuronal systems, including increased Ca^{2+} entry, transmitter release, and long-term potentiation of synaptic efficacy²³⁻²⁵.

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- Reuter, H. *Nature* **301**, 569–574 (1983).
- Siegelbaum, S. A. & Tsien, R. W. *Trends Neurosci.* **6**, 307–312 (1983).
- Tsien, R. W. in *Neuromodulation: The Biochemical Control of Neuronal Excitability* (eds Kaczmarek, L. K. & Levitan, I. B.) 206–242 (Oxford University Press, New York, 1986).
- Rane, S. G. & Dunlap, K. *Proc. natn. Acad. Sci. U.S.A.* **83**, 184–188 (1986).
- Levitan, I. B. *J. memb. Biol.* **87**, 177–190 (1986).
- Farley, J. & Auerbach, S. *Nature* **319**, 220–223 (1986).
- Kaczmarek, L. K., Strong, J. A. & Kauer, J. A. *Prog. Brain Res.* (in the press).
- Reuter, H., Stevens, C. F., Tsien, R. W. & Yellen, G. *Nature* **297**, 501–504 (1982).
- Cachelin, A. B., dePeyer, J. E., Kokubun, S. & Reuter, H. *Nature* **304**, 462–464 (1983).
- Bean, B. P., Nowycky, M. C. & Tsien, R. W. *Nature* **307**, 371–375 (1984).
- Brum, G., Osterrieder, W. & Trautwein, W. *Pflügers Arch. ges. Physiol.* **401**, 11–18 (1984).
- Tsien, R. W. *et al. J. molec. Cell Cardiol.* **18**, 691–711 (1986).
- DeRiemer, S. A., Strong, J. A., Albert, K. A., Greengard, P. & Kaczmarek, L. K. *Nature* **313**, 313–316 (1985).
- DeRiemer, S. A., Greengard, P. & Kaczmarek, L. K. *J. Neurosci.* **5**, 2672–2676 (1985).
- Nishizuka, Y. *Science* **225**, 1365–1370 (1984).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85–100 (1981).
- Hess, P., Lansman, J. B. & Tsien, R. W. *J. gen. Physiol.* **88**, 293–319 (1986).
- Fenwick, E. M., Marty, A. & Neher, E. *J. Physiol., Lond.* **331**, 599–635 (1982).
- Fukushima, Y. & Hagiwara, S. *J. Physiol., Lond.* **358**, 255–284 (1985).
- Hagiwara, S. & Byerly, L. *Trends Neurosci.* **6**, 189–193 (1983).
- Siegelbaum, S. A., Camardo, J. S. & Kandel, E. R. *Nature* **299**, 413–417 (1982).
- Blumberg, P. M. *et al. Biochem. Pharmacol.* **33**, 933–940 (1984).

- Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J. & Routtenberg, A. *Science* **231**, 587–589 (1986).
- Malenka, R. C., Madison, D. V. & Nicoll, R. A. *Nature* **331**, 175–177 (1986).
- Zurgil, N. & Zisapel, N. *FEBS Lett.* **185**, 257–261 (1985).
- Kaczmarek, L. K., Finbow, M., Revel, J. P. & Strumwasser, F. *J. Neurobiol.* **10**, 535–550 (1979).

Involvement of dihydropyridine receptors in excitation–contraction coupling in skeletal muscle

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The transduction of action potential to muscle contraction (E–C coupling) is an example of fast communication between plasma membrane events and the release of calcium from an internal store, which in muscle is the sarcoplasmic reticulum (SR)¹. One theory is that the release channels of the SR are controlled by voltage-sensing molecules or complexes, located in the transverse tubular (T)-membrane, which produce, as membrane voltage varies, 'intramembrane charge movements'², but nothing is known about the structure of such sensors. Receptors of the Ca-channel-blocking dihydropyridines present in many tissues³, are most abundant in T-tubular muscle fractions^{4,5} from which they can be isolated as proteins. Fewer than 5% of muscle dihydropyridines are functional Ca channels⁶; there is no known role for the remainder in skeletal muscle physiology. We report here that low concentrations of a dihydropyridine inhibit charge movements and SR calcium release in parallel. The effect has a dependence on membrane voltage analogous to that of specific binding of dihydropyridines⁶. We propose specifically that the molecule that generates charge movement is the dihydropyridine receptor.

Transient increases in myoplasmic $[\text{Ca}^{2+}]$ ('Ca transients') associated with pulse depolarization of single fibres of frog semitendinosus, were obtained by changes in light absorbed by a calcium-sensitive dye in the myoplasm⁷. Figure 1a illustrates the effect of nifedipine on Ca transients, elicited at a holding potential (h.p.) of -70 mV (left) or -100 mV (right). In this and four other experiments nifedipine caused a large reduction in the Ca transient at h.p. = -70 mV (Fig. 1a trace 4) and a much smaller reduction at h.p. = -100 mV (Fig. 1a trace 3). That the effect was small at h.p. = -100 mV may explain a recent negative report on effects of nifedipine on contraction⁸.

The Ca transients are the result of release of Ca from the SR and removal of calcium by various cell structures and the dye. To separate the effects of nifedipine on release alone, we derived from the Ca transients the 'rate of release'⁹ or release flux¹⁰ (Fig. 1b), representing the flux of calcium into the myoplasm per unit time and unit myoplasmic water volume. The effects on release flux were much greater, in both relative and absolute terms, at h.p. = -70 mV; in this and two other experiments 500 nM nifedipine caused a reduction of about 70% after 20 min exposure (Fig. 1b, record 4) and greater than 80% reduction after 30 min (two fibres, Fig. 3b). At h.p. = -100 mV after more than 20 min the effect was a 6% reduction (s.e.m. = 6%, $n = 5$).

The 'release' waveform lumps together all contributions that increase myoplasmic Ca^{2+} , including release from the SR and calcium influx via the T-membrane Ca channel¹¹. In principle, the decrease in the release waveform caused by nifedipine could be due to blockage of the T-membrane Ca channel and abolition of calcium influx through it. We estimated the effect of nifedipine on the T-membrane Ca current by subtracting total current in nifedipine from the total membrane current. This difference was negligible compared with the reduction of release flux caused

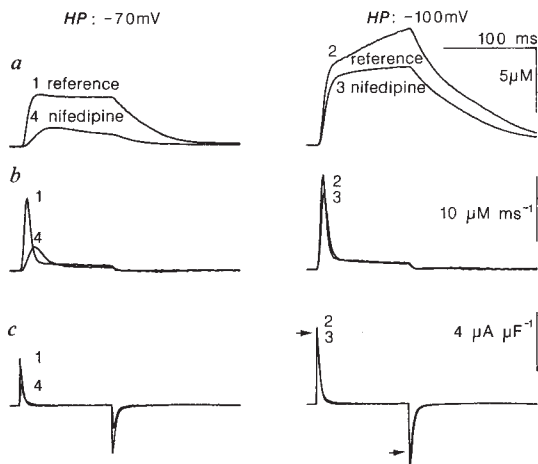


Fig. 1 *a*, Time course of myoplasmic $[\text{Ca}^{2+}]$ derived from absorbance changes in the presence of the dye Antipyrilazo III^{28,29} in a cut skeletal fibre under voltage clamp. A detailed description of the technique has been published³⁰. *b*, Rate of release⁹ or release flux¹⁰ records derived from the records in *a*. *c*, Records of asymmetric membrane current ('charge movement'), corresponding to the calcium records in *a* and *b*.

Methods. In *a*, the cut ends of a fibre segment were exposed to an internal solution containing 0.8 mM of Antipyrilazo III (K&K Labs). The central portion of the fibre was voltage clamped and polarized to h.p. = -70 mV (left-hand records) or -100 mV (right-hand records) in a double vaseline gap chamber with a glass bottom. Light from a tungsten-halogen 100 W bulb was filtered to eliminate wavelengths <650 nm and focused on the fibre. The transmitted light was collected with an immersion objective and its intensity recorded at 700 nm and 850 nm. The intensity record at 700 nm was corrected for intrinsic changes in absorbance (recorded at 850 nm) and processed to derive time course of $[\text{Ca}^{2+}]$ ³⁰. The applied pulse went in all cases shown from -70 mV to 0 mV (100 ms). Records 1 and 2 were obtained in reference external solution, 3 and 4 after 20 min in the same solution plus 500 nM high-purity nifedipine (gift from Pfizer Laboratories). The records were obtained in the sequence 1,2,3,4: other experiments with different sequences gave similar results. In *c*, records were obtained as differences between the total membrane current during the test pulse and a control current recorded previously with the fibre held at h.p. = 0 mV during a pulse to +70 mV¹². A sloping baseline was fitted to the last 50 ms of the ON and OFF portions of the difference and subtracted. Arrows, peaks of record 3 at ON and OFF. Fibre diameter, 55 μm ; length under clamp, 540 μm . Calibration bar, 30 nA or 32 $\mu\text{A cm}^{-2}$ surface area. Temperature 11 °C. Composition of solutions: tetraethylammonium-sulphate, 75 mM; Cs_2SO_4 , 5 mM; caesium Tris-maleate buffer, 5 mM; CaSO_4 , 10 mM; tetrodotoxin (TTX), 10^{-7} g ml^{-1} ; 3-4 diaminopyridine, 1 mM. Internal solution (in end compartments of the double vaseline gap): caesium glutamate, 108; MgCl_2 , 5.5; sodium Tris-maleate, 4.5; caesium Tris-maleate, 13.2; EGTA, 0.1; CaCl_2 0.0082; Na_2ATP , 5; glucose, 5; Antipyrilazo III, 0.8; pH 7.0. Data acquisition and pulse generation performed with a laboratory computer (LSI-11/23 PLUS, DEC) with A/D and D/A conversion boards (DT 3382 and D7 3371, Data Translation)¹².

by the drug, indicating that nifedipine actually reduces Ca release from the SR.

The effect is probably mediated by a direct action of the drug on the voltage sensor of E-C coupling. Figure 1c shows the charge movement records that correspond to the calcium records in *a* and *b*; there is a substantial effect of nifedipine at h.p. = -70 mV and a small effect at h.p. = -100 mV. A more detailed exploration of effects on charge movement at h.p. = -70 mV (Fig. 2) showed that charge moved during a depolarizing pulse to 0 mV was much smaller after 10 min in 100 nM nifedipine (records 3 and 4, Fig. 2a).

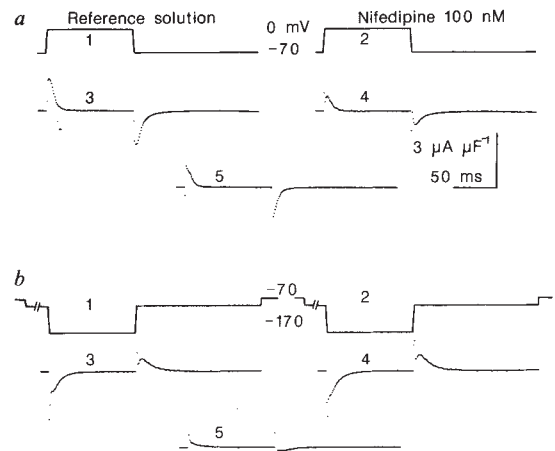


Fig. 2 Effect of nifedipine on intramembrane charge movement. Left, records in reference solution; right, records in 100 nM nifedipine. h.p. = -70 mV. *a*, Recorded membrane voltage (records 1 and 2) and charge movements (records 3 and 4). Record 5, total current during the test pulse in reference solution minus total current during the test pulse in nifedipine (record 3 minus record 4). *b*, Charge movement records during test pulses from -100 mV (prepulse level) to -170 mV. The control current was the same as in *a*. Record 5, total current during the test pulse in reference solution minus total current in nifedipine (record 3 minus record 4). Same solutions as in Fig. 1. Temperature, 7 °C. Fibre diameter, 75 μm . Calibration bar, 33 nA or 26 $\mu\text{A cm}^{-2}$ surface area.

The records of charge movement are obtained by subtracting a ('control') linear capacitive current from the total current recorded during the test pulse. Conventionally, control currents are determined with negative pulses from -100 mV. We recently demonstrated¹² that records obtained with positive pulses from a h.p. = 0 mV better satisfy the criteria of linearity required for control pulses. Using these controls it is clear that charge moves in the range of voltages negative to -100 mV (Fig. 2b). Charge movement in reference solution (Fig. 2b record 3) or nifedipine (record 4) for a pulse from -100 mV to -170 mV was measured. Surprisingly, in 8 out of 9 fibres studied the charge movement increased in the presence of nifedipine. The average increase in charge moved for a pulse from -100 mV to -170 mV was 3.7 nC μF^{-1} (0.87 s.e.m.). Thus, nifedipine not only decreases the charge that moves positive to -70 mV (refs 13, 14) but also increases the charge that moves negative to -100 mV. The effects are similar in this regard to those of prolonged depolarization¹², which include an increase in charge mobile at large negative potentials, probably corresponding to transitions between inactivated (or nifedipine-bound) states of the voltage sensor¹².

Table 1 summarizes data on concentration-dependence. Substantial effects were observed even at 10 nM, provided that the drug was perfused continuously for 20 min or more. Figure 3a and b represents the time course of onset and recovery of the effect on peak release flux and total charge moved during a pulse to 0 mV. The effects on charge movement and calcium release were essentially cotemporal. The onset was slow and, especially at higher concentrations, the effects still increased after 30 min of drug exposure.

The experiment in Fig. 3b demonstrates photoinactivation of the drug¹⁵. After 15 min nifedipine treatment of the specimen, filters that normally eliminate incident light of short wavelength were removed at times (arrows) for brief intervals. This caused rapid but incomplete recovery of both release and charge. The magnitude of the recovery and subsequent relapse was comparable for both variables.

In Fig. 3c, charge movement elicited with pulses positive to h.p. = -70 mV is plotted against peak release in several fibres in reference solution and exposed to different nifedipine con-

Table 1 Effects of three concentrations of nifedipine on charge movement and peak release flux

[Nifedipine] (nM)	10	100	500	500
h.p. (mV)	-70	-70	-70	-100
n (fibres)	3	4	2	5
Reduction in charge movement (%)	36 (33-38)	62 (33)	67 (65-69)	29 (7.0)
Reduction in peak release (%)	37 (14-59)	67 (8.7)	84 (79-89)	6 (6.0)

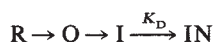
Effects calculated from an average value before nifedipine and the value in nifedipine at the longest exposure time available (between 18 min and 32 min). Average reductions are followed by the s.e.m. (or the range, whenever the sample number is ≤ 3) in parentheses. Charge movement figures are averages of ON and OFF. All changes were significant ($P < 0.05$) except that of peak release at h.p. = -100 mV. The reductions of the ON and OFF charges at h.p. = -100 mV (22 and 37% respectively) were significantly different. Temperature, 7-11 °C. Test pulse from -70 to 0 mV.

centrations. The regression line is derived from the points in reference solution. This line describes well the results in nifedipine and passes very close to the centre of mass of the points in nifedipine. Thus, the effect of nifedipine on Ca release seems well explained by its effect on charge movement. However, the regression line does not go through the origin, suggesting that a minor fraction of the charge is not directly involved in signalling release.

Two main results have been reported here: (1) nifedipine inhibits Ca release from the SR, probably by an effect on intramembrane charge movement; (2) the effect is greater at h.p. = -70 mV than at h.p. = -100 mV.

The first result indicates that nifedipine binding molecules are involved in E-C coupling. The second result suggests that these molecules are the high affinity dihydropyridines (DPRs), as binding to DPRs depends on h.p. in a similar way⁶. DPRs have been identified in various membrane preparations³ but in T-tubular fractions, which constitute the richest source¹⁶, their density is 20-50 times that of membrane Ca channels⁶. In addition to voltage dependence of binding, several of their properties are consistent with the hypothesis that DPRs are the voltage sensors of E-C coupling or closely interact with them in a protein complex. (1) Their density is roughly consistent with that of intramembrane charge movement particles. The density of DPRs in frog muscle is ~ 230 sites μm^{-2} of T-tubular membrane⁶. The density of charge movement sites was estimated at 500-600 sites μm^{-2} of T-tubular plus surface membrane¹⁷. (2) Other drugs, like D-600¹⁵ and verapamil¹⁶ interact specifically with the DPRs, perhaps at a different binding site on the same molecule. D-600 both inhibits contractility¹⁸ (presumably by preventing Ca release) and prevents charge movement¹⁹ at concentrations consistent with its affinity for DPRs²⁰. (3) Mice with muscular dysgenesis²¹ lack E-C coupling²². These mice have a very reduced density of DPRs²³ and lack slow Ca currents²⁴.

From the average value of the effects of 10 and 100 nM nifedipine on release flux a dissociation constant of about 32 nM results for a simple one-site reaction. The dependence of the effect on h.p. suggests that the high affinity is a property of conformations of the receptor different from its resting state, as proposed for Ca channels^{25,26}. The simple model:



in which nifedipine (N) cannot bind to the resting or open states of the sensor (R, O) but only to the inactivated state (I), with dissociation constant K_D , predicts the relationship²⁵

$$K_{app}(h.p.) = K_D/[1 - h_{\infty}(h.p.)]$$

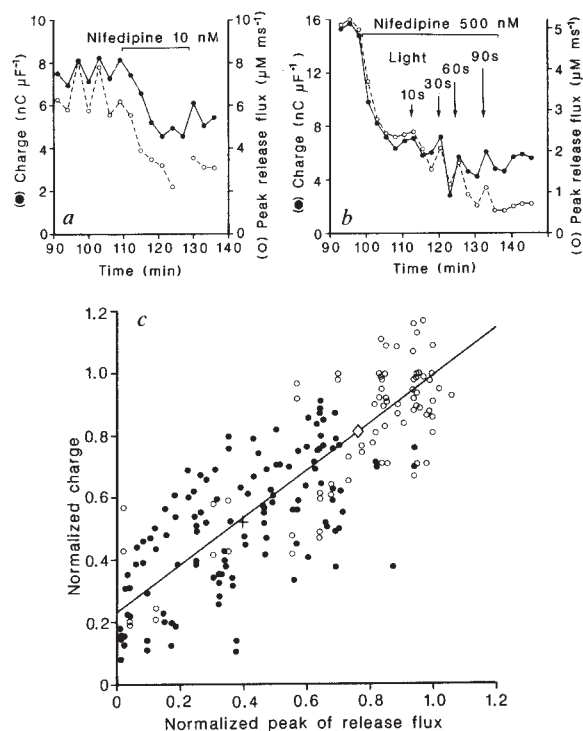


Fig. 3 *a, b*, Maximum value of release flux (○) and area under the charge movement transient, average of ON and OFF (●), for pulses from h.p. (-70 mV) to 0 mV in two different fibres exposed to 10 nM (*a*) and 500 nM nifedipine (*b*) during the interval marked by the bars. Temperature, 11 °C. In *a* the release value at 128 min was not measured. In *b* the filter that normally blocks short wavelength radiation was removed from the path of the incident light (used to monitor calcium) at the times indicated by arrows for intervals of 10, 30, 60 and 90 seconds. *c*, Charge moved during depolarizing pulses plotted against peak release flux. Data from 10 fibres. Each fibre was first pulsed in reference solution (○) later in a nifedipine-containing solution (●), at concentrations ranging from 100 nM to 10 μM . The depolarizing pulses were applied from a -70 mV holding potential to different test potentials (-40 mV to +30 mV); about the same voltage pulses were applied in both solutions. Peak release flux and charge movement values, both in reference solution and in nifedipine, were divided by a normalization value, calculated for each individual fibre as the average of the values obtained in reference solution with pulses to 0 mV or greater. The regression line calculated for the values in reference solution has slope 0.762 (0.104 s.d.), intercept 0.231 (0.048 s.d.) and correlation coefficient 0.822. The corresponding values in nifedipine (regression line not shown) are slope 0.590 (0.060 s.d.), intercept 0.285 (0.028 s.d.) and correlation coefficient 0.655. Neither slopes nor intercepts are significantly different. Centres of mass (◇) in reference solution and (+) in nifedipine are also shown.

where K_{app} (h.p.) is the apparent value of the dissociation constant at a holding potential at which the steady value of the inactivation parameter is h_{∞} (h.p.). This model predicts K_{app} (-70 mV) to be four to five times greater than K_D , as the steady inactivation of release at -70 mV is 0.2 to 0.25 (compare records 1 and 2 in Fig. 1*b*). Therefore the estimate of 32 nM obtained for K_{app} in these experiments corresponds to a K_D of ~ 7 nM; by comparison the dissociation constant of nifedipine binding is 4 nM (rabbit muscle¹⁶).

Other possible sites of action of nifedipine should be considered; it is possible but unlikely that depletion of the SR and secondary reduction in its ability to release calcium mediate the nifedipine effects; a rapid recovery after tens of seconds of intense illumination (Fig. 3*b*) seems inconsistent with depletion as the main mechanism. That nifedipine has mainly a direct intracellular effect on the Ca-release channel of the SR, beyond

the voltage sensor, or merely an effect of non-specific partition into membranes is also unlikely because of the parallel effect on charge movement and the h.p.-dependence of the effects.

We have described here effects of nifedipine consistent with an involvement of the high affinity DPRs in E-C coupling. DPRs are generally thought of as Ca channels. It is possible that they are channel-like proteins, not necessarily conducting, that perform the voltage sensing function and are coupled to the SR release pathway by unknown means, involving perhaps a mediator²⁷.

Part of these results have been communicated in abstract form¹³. We thank Dr Enrico Stefani for help and encouragement, Dr G. Pizarro for help with experiments, Drs T. DeCoursey, F. Cohen, R. Eisenberg and W. K. Chandler for discussions and Ms. L. Vaughn for careful typing. Supported by NIH grant AM32808.

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1. Ebashi, S. *A. Rev. Physiol.* **38**, 293-314 (1976).
2. Schneider, M. F. & Chandler, W. K. *Nature* **242**, 244-246 (1973).
3. Janis, R. A. & Triggler, D. J. *Drug Dev. Res.* **4**, 257-274 (1984).

4. Curtis, B. M. & Catterall, W. A. *Biochemistry* **23**, 2113-2118 (1984).
5. Borsotto, M., Norman, R. I., Fosset, M. & Lazdunski, M. *Eur. J. Biochem.* **142**, 449-455 (1984).
6. Schwartz, L. M., McCleskey, E. W. & Almers, W. *Nature* **314**, 747-751 (1985).
7. Kovacs, L., Rios, E. & Schneider, M. F. *Nature* **279**, 391-396 (1979).
8. McCleskey, E. W. *J. Physiol., Lond.* **361**, 231-249 (1985).
9. Melzer, W., Rios, E. & Schneider, M. F. *Biophys. J.* **45**, 637-641 (1984).
10. Melzer, W., Rios, E. & Schneider, M. F. *Biophys. J.* (in the press).
11. Beatty, G. N. & Stefani, E. *J. Physiol., Lond.* **260**, 27P (1976).
12. Brum, G. & Rios, E. *J. Physiol., Lond.* (in the press).
13. Rios, E., Brum, G. & Stefani, E. *Biophys. J.* **49**, 13a (1986).
14. Lamb, G. D. *J. Physiol., Lond.* **376**, 85 (1986).
15. Morad, M., Goldman, Y. E. & Trentham, D. R. *Nature* **304**, 635-638 (1983).
16. Fosset, M., Jaimovich, E., Delpont, E. & Lazdunski, M. *J. biol. Chem.* **258**, 6086-6092 (1983).
17. Chandler, W. K., Rakowski, R. F. & Schneider, M. F. *J. Physiol., Lond.* **254**, 245-283 (1972).
18. Eisenberg, R. S., McCarthy, R. T. & Milton, R. L. *J. Physiol., Lond.* **341**, 495-595 (1983).
19. Hui, C. S., Milton, R. L. & Eisenberg, R. S. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2582-2585 (1984).
20. Toll, L. *J. biol. Chem.* **255**, 13189-13192 (1982).
21. Gluecksohn-Waelsch, S. *Science* **142**, 1269-1276 (1963).
22. Klaus, M. M., Stylianou, P. S., Rapalus, J. M., Briggs, R. T. & Powell, J. A. *Dev. Biol.* **99**, 152-165 (1983).
23. Pincon-Raymond, M., Rieger, F., Fosset, M. & Lazdunski, M. *Neurosci. Abstr.* **10**, 202 (1984).
24. Beam, K. G., Knudson, C. M. & Powell, J. A. *Nature* **320**, 168-170 (1986).
25. Bean, B. P. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6388-6392 (1984).
26. Sanguinetti, M. C. & Kass, R. S. *Circulation, Res.* **55**, 336-348 (1984).
27. Vergara, J., Tsien, R. Y. & Delay, M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6352-6356 (1985).
28. Scarpa, A., Brinley, F. J. & Dubyak, G. *Biochemistry* **17**, 1378-1386 (1978).
29. Rios, E. & Schneider, M. F. *Biophys. J.* **36**, 607-621 (1981).
30. Kovacs, L., Rios, E. & Schneider, M. F. *J. Physiol. Lond.* **343**, 161-196 (1983).

T γ protein is expressed on murine fetal thymocytes as a disulphide-linked heterodimer

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During the search for genes coding for the mouse α and β subunits of the antigen-specific receptor of mouse T cells we encountered a third gene, subsequently designated γ^1 . This gene has many properties in common with the α and β genes^{1,2}; somatic assembly from gene segments that resemble the gene segments for immunoglobulin variable (V), joining (J) and constant (C) regions; rearrangement and expression in T cells and not in B cells; low but distinct sequence homology to immunoglobulin V, J and C regions; other sequences that are reminiscent of the transmembrane and intracytoplasmic regions of integral membrane proteins; and a cysteine residue at the position expected for a disulphide bond linking two subunits of a dimeric membrane protein. Despite these similarities the γ gene also shows some interesting unique features. These include a relatively limited repertoire of the germ-line gene segments²⁻⁵, more pronounced expression at the RNA level in immature T cells such as fetal thymocytes⁶⁻⁸ and an apparent absence of in-frame RNA in some functional, $\alpha\beta$ heterodimer-bearing T cells or cultured T clones and hybridomas^{9,10}. To understand the function of the putative γ protein it is essential to define the cell population that expresses this protein. To this end we produced a fusion protein composed of *Escherichia coli* β -galactosidase and the γ -chain (hereafter referred to a β -gal- γ) using the phage expression vector λ gt11 (ref. 11) and raised rabbit antisera against the γ determinants. Using the purified anti- γ antibody we detected a polypeptide chain of relative molecular mass 35,000 (M_r 35K) on the surface of 16-day old fetal thymocytes. The γ -chain is linked by a disulphide bridge to another component of 45K. No such heterodimer was detected on the surface of a cytotoxic T lymphocyte (CTL) clone 2C12 from which an in-phase γ cDNA clone was originally isolated.

We inserted various parts of the γ cDNA clone pHDS4/203 which contains an in-phase $V_2J_2C_2$ sequence (see ref. 4 and 9 for the nomenclature of mouse γ gene segments) into the *EcoRI*

cloning site of the phage expression vector λ gt11 and determined the proportion of the β -gal- γ fusion proteins synthesized in the various lysogens by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (data not shown). We then chose the phage construct λ gt11- γ^1 depicted in Fig. 1a because it gave the highest yield of the fusion protein (~4% of the total protein upon induction by isopropyl-thio- β -D-galactoside (IPTG). This γ insert contains almost all the V_2J_2 region and part of the C region and should code for 15K of the γ polypeptide chain. Figure 1b shows Coomassie Blue-stained gels of the lysates prepared from the λ gt11- γ^1 lysogen without and with induction by IPTG (lanes 2 and 3). The lysate of the λ gt11 lysogen with induction is also shown (lane 1). It is apparent from these gel patterns that the 131K band observed in lane 3 is the expected β -gal- γ fusion protein.

The fusion protein was purified by centrifugation followed by preparative SDS-PAGE. As shown in Fig. 1b lanes 4-7, the fusion protein can be thus purified close to homogeneity. After removing most of the bound SDS by extensive dialysis, the purified fusion protein mixed with complete Freund's adjuvant was injected subcutaneously into rabbits. Anti- γ -chain antibodies were purified from the sera of hyperimmunized rabbits by first removing anti- β -galactosidase antibody on a β -galactosidase immunoabsorbent and then applying the flow-through to a second immuno-adsorbent composed of the β -gal- γ fusion protein conjugated with Sepharose beads.

To demonstrate that the material eluted from the fusion protein immunoabsorbent indeed contains anti- γ activity we prepared γ protein or fragments of it by two different methods. First, 1 μ g of $V_2J_2C_2$ γ RNA was prepared *in vitro* using the SP6 transcription system (Fig. 2a)^{13,14}. The RNA was then translated *in vitro* into the γ protein using a rabbit reticulocyte lysate. Figure 2b lane 1 shows a SDS-PAGE display of the ³⁵S-labelled γ -chain that has been admixed before electrophoresis with the rabbit β globin similarly synthesized *in vitro*. That the purified putative anti- γ antibodies but not the anti β -galactosidase antibody indeed contain anti- γ antibody is demonstrated by the specific immunoprecipitation of the *in vitro* synthesized γ -chain by the former (Fig. 2b, lanes 2-5).

Alternatively we prepared a fusion protein composed of mouse immunoglobulin κ chain and the γ -chain constant region by transfecting a myeloma cell J558L with an appropriately constructed chimaeric plasmid. Thus the major C_γ exon and its flanking sequences were inserted in the major intron of a κ light chain gene (Fig. 3a). The myeloma cells that have been stably transfected with the plasmid secrete, albeit in small amounts, the $V_\kappa C_\gamma C_\kappa$ three-domain polypeptide chain into the medium

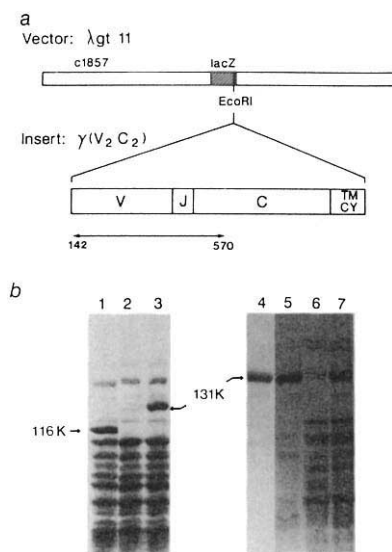


Fig. 1 Synthesis and purification of β -galactosidase- γ fusion protein. *a*, Construction of the chimaeric phage λ gt11- γ 1. A 429-bp DNA fragment coding for the entire *VJ* region and part of the *C* region of the γ cDNA clone pHDS4/203 (ref. 1) was inserted into the *EcoRI* site of the phage λ expression vector λ gt11 (ref. 11). *b*, Purification of the fusion protein. Aliquots of the lysates prepared from λ gt11 or λ gt11- γ 1 lysogens or fractions thereof were analysed by SDS-PAGE. The gel was stained with Coomassie Blue. Lane 1, λ gt11 lysogen induced by IPTG; lane 2, λ gt11- γ 1 lysogen uninduced; lanes 3 and 7, induced λ gt11- γ 1 lysogen; lanes 5 and 6, pellet and supernatant fractions (respectively) after a low speed centrifugation of the lysate of the induced λ gt11- γ 1 lysogens; lane 4, fusion protein eluted from a preparative PAGE run.

Methods. $V_2J_2C_2$ γ cDNA clone pHDS4/203 was digested with *PvuII* and *BstXI* and the 429-bp fragment (nucleotide positions 142-570; see ref. 3 for the numbering system used) was purified, blunt-ended by *T*₄ polymerase, and ligated into the unique *EcoRI* site of λ gt11 DNA using *EcoRI* linkers. The recombinant DNA was packaged *in vitro* using the λ phage packaging mix (Promega) and the packaged phages were plated on *E. coli* Y1088 (sup F⁺). The phages containing the γ DNA sequence in the appropriate orientation were identified by plaque hybridization followed by restriction enzyme analysis of the purified phage DNA. The lysogens prepared with these phages as well as the vector phage λ gt11 were grown and the β -galactosidase or the β -gal- γ fusion gene was induced with IPTG by the standard procedure¹¹. The bacterial cells were collected in a lysis buffer (0.2 M Tris-HCl pH 7.6, 0.2 M NaCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol (DTT), 1 mM PMSF (phenylmethylsulphonyl fluoride) and 100 units ml⁻¹ Aprotinin and disrupted by freezing and thawing, then sonication. The lysate was centrifuged at 20,000g for 40 min at 4°C. The pellet was washed once with the above buffer. For the preparative SDS-PAGE the washed pellet was suspended in the lysis buffer. The proteins were dissolved by addition of one volume of 2-fold concentrated SDS-PAGE sample buffer (160 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 4 mM EDTA, 10% β -mercaptoethanol) and electrophoresed in 6% cylindrical acrylamide gels of 15 mm diameter. The 131K protein was electroeluted in the electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and dialysed against a buffer containing 50 mM Tris-HCl pH 7.5, 2.5 mM octylglucoside, 0.1 mM PMSF, followed by the same buffer without octylglucoside.

along with the endogenous λ light chain (Fig. 3b, lane 1). The results shown in the rest of Fig. 3b show that the fusion protein is specifically precipitated with the serum of the rabbit hyperimmunized with the β -gal- γ fusion protein. These results not only confirm the rabbit serum contains anti- γ -antibody but also demonstrate that at least some of this antibody is directed against the *C*_γ region.

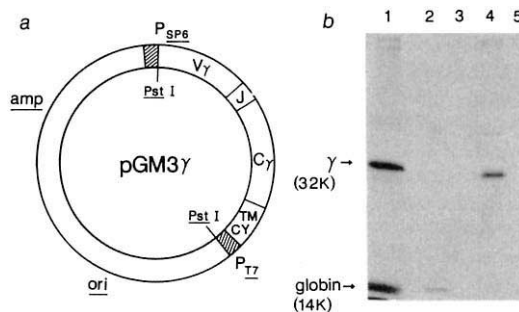


Fig. 2 Immunoprecipitation of the *in vitro*-synthesized γ polypeptide chain by the putative anti- γ antibody. *a*, Construction of a plasmic, pGM3 γ , for the *in vitro* transcription by SP6 RNA polymerase. The γ cDNA pHDS4/203 was subjected to limited digestion with *PstI* and the 1372-bp fragment (nucleotide positions 43-1414 (ref. 3)) containing the entire translated region and some flanking untranslated regions of the γ cDNA was ligated to the *PstI* site of pGEM3 (Promega). This vector carried the transcription promoter for SP6 RNA polymerase just upstream of the *PstI* site. *b*, A SDS-PAGE autoradiogram of the immunoprecipitate of the *in vitro*-synthesized γ polypeptide chain. The ³⁵S-labelled γ protein synthesized *in vitro* was mixed with the similarly synthesized rabbit β globin (lane 1) and precipitated with a rat anti-rabbit-globin (lane 2), a normal rat serum (lane 3), the anti- γ fraction (lane 4) and anti- β -galactosidase fraction (lane 5) of the serum of the rabbit hyperimmunized with the β -gal- γ fusion protein.

Methods. pGM38 DNA was linearized with *Bam*HI transcribed by SP6 RNA polymerase (Promega) as described^{13,14}. The template DNA was digested with RNase-free DNase and the RNA was extracted by a phenol/chloroform mix (1:1), precipitated by 2.5 volumes of ethanol, and extensively washed with 80% ethanol. The purified γ RNA was translated into ³⁵S-labelled protein using the lysate of rabbit reticulocytes (Promega) according to the procedures recommended by the supplier. In order to raise the anti- β -gal- γ fusion protein, rabbits were immunized subcutaneously with the 1 mg purified fusion protein with complete Freund's adjuvant. After three weeks the rabbits were boosted with 1 mg of the fusion protein with incomplete Freund's adjuvant and bled 7 d later. The immunoglobulin was precipitated from the antisera with one volume of saturated ammonium sulphate, dialysed extensively against a phosphate-buffered saline (PBS) and passed through a column composed of β -galactosidase (Sigma)-conjugated Sepharose 4B. The material adsorbed was eluted from the column with 0.2 M glycine-HCl buffer (pH 2.6) (anti- β -galactosidase fraction). The effluent was passed through a second affinity column containing the purified fusion protein and the material adsorbed (anti- γ fraction) was similarly eluted. Immunoprecipitation was carried out by standard procedures using protein A-Sepharose.

We next investigated whether a bona fide γ protein could be detected on murine cells of the T-lineage. We chose fetal thymocytes and a CTL clone 2C because these cells were previously shown to contain a relatively high level of γ RNA^{1,6-8}. Furthermore the 2C γ RNA was shown to be in-phase¹. Cells were surface-labelled with Na¹²⁵I and lactoperoxidase and lysed in 1% NP40. The lysates were subjected to immunoprecipitation with the affinity-purified anti- γ antibody. As a negative control, the same lysates were immunoprecipitated with the anti- β -galactosidase antibody also affinity-purified from the same hyperimmune rabbit serum from which the anti- γ antibody was purified. The immunoprecipitate was analysed by SDS-PAGE under non-reducing conditions in one dimension and then under reducing conditions in the second. Under these conditions those cell-surface proteins containing interchain disulphide linkages migrate differently in the first and second dimensions and are usually displayed in the region below the diagonal line¹⁵. As shown in Fig. 4a, two off-diagonal spots of 45K and 35K are

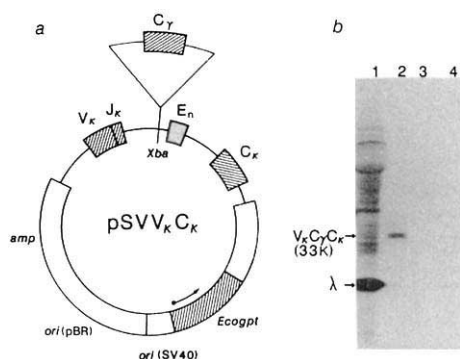


Fig. 3 Immunoprecipitation by the rabbit anti- γ antiserum of the $V_{\kappa}C_{\kappa}$ polypeptide chain secreted from a myeloma transfectant. *a*, Construction of the recombinant plasmid pSV $V_{\kappa}C_{\kappa}$ containing the C_{κ} exon within the major intron of a rearranged κ gene. The $EcoRI$ - $BamHI$ DNA fragment containing the entire rearranged κ gene was cloned from a mouse myeloma LPC-1 and inserted to a plasmid pSV2gpt²⁶. The $HindIII$ DNA fragment containing major C_{κ} exon² and its flanking sequences was then inserted at the unique $XbaI$ site between the $V_{\kappa}J_{\kappa}$ exon and the enhancer (E_n). *b*, An SDS-PAGE autoradiogram of the immunoprecipitate of the $V_{\kappa}C_{\kappa}$ protein. ³⁵S-methionine-labelled supernatant of the culture of myeloma J558L stably transfected with pSV $V_{\kappa}C_{\kappa}$ (lane 1) was immunoprecipitated by goat anti-mouse κ light chains (lane 2), anti- β -galactosidase fraction (lane 3) or the anti- γ fraction of the rabbit serum obtained after hyperimmunization with the β -gal- γ fusion protein. J558L secretes a λ light chain in large amounts and some of it is carried over non-specifically in the immunoprecipitate.

Methods. A mouse myeloma J558L was transfected with pSV $V_{\kappa}C_{\kappa}$ by the protoplast fusion method as described previously²⁷. The culture of stable transfectant D8.2 was fed with 0.4 mCi per ml ³⁵S-methionine (Amersham) for 5 h. The protein in the culture supernatant was denatured and partially renatured as described²⁰ and was subjected to immunoprecipitation and SDS-PAGE.

present in the autoradiogram obtained with the lysate prepared from day 16 fetal thymocytes and the anti- γ antibody. No such spots were detectable when the anti β -galactosidase antibody was used (Fig. 4b).

The results of the analysis of the CTL clone 2C are shown in Fig. 4d. It seems that neither the 45K nor the 35K 'off-diagonal' component is present on the surface of this CTL clone.

The M_r of the $V_2J_2C_2$ γ -chain predicted by the nucleotide sequence is 33K and the chain should have no N -linked carbohydrate. Thus the measured molecular mass, 35K, of the smaller of the two 'off-diagonal' components observed in the fetal thymocytes is in good agreement with the predicted value. Preliminary experiments in which lysate was treated with dithiothreitol, immunoprecipitated and analysed by one-dimensional SDS-PAGE show a specific 35K component in the precipitates obtained with the anti- γ antibody which is competed out when excess purified β -gal- γ fusion protein is added to the lysate before immunoprecipitation (data not shown). Thus it seems that the thymocyte population derived from 16-day-old mouse fetuses contains cells that bear on the surface a γ -chain linked by one or more disulphide bonds to a 45K component.

As the anti- γ antibody was prepared by using as the immunogen the polypeptide encoded by a part of $V_2J_2C_2$ cDNA (see Fig. 1a), we tacitly assumed that the γ chain represented by the 35K component is of the $V_2J_2C_2$ type. But three more V gene segments, V_4 , V_5 and V_6 , are known to be expressed at least at the RNA level in fetal thymocytes in combination with another JC pair, J_1C_1 ^{4,10,11}. Furthermore another γ gene, $V_1J_4C_4$, was recently reported¹⁷. As J_1C_1 and V_2 are highly homologous to J_2C_2 (94% homology at the protein level) and V_1 (87% homology), respectively, it is possible that the anti- $V_2J_2C_2$ antibodies used in this study crossreact with the γ -chain

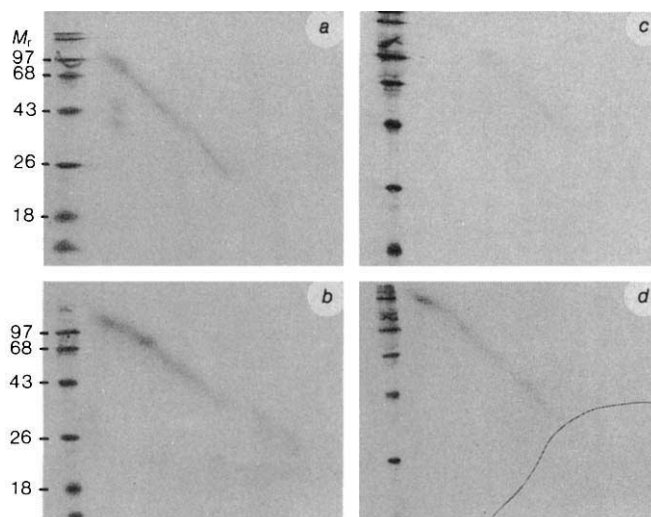


Fig. 4 Two-dimensional PAGE analysis of immunoprecipitated γ ¹²⁵I-labelled proteins from fetal thymocytes and CTL clone 2C. *a*, *b*, Lysates of fetal thymocytes from day 16 embryos immunoprecipitated by the anti- γ and anti- β -galactosidase fractions, respectively. *c*, *d*, Lysates of CTL clone 2C immunoprecipitated by anti- γ and anti- β -galactosidase, respectively. PAGE was performed under nonreducing conditions from left to right and reducing conditions from top to bottom.

Methods. Fetal thymi were dissected from the embryos of the day 16 pregnant mothers. The thymocytes were suspended in PBS and surface-labelled with ¹²⁵I using lactoperoxidase. The cells were washed three times with PBS, suspended in cold NP40 buffer (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM PMSF and 1% Nonidet P40) and disrupted by vigorous shaking. The lysates were subjected to immunoprecipitation and the immunoprecipitates were electrophoresed under nonreducing conditions through 10% acrylamide gels formed in a glass tube of internal diameter 1.5 mm. The gel was then removed from the glass tube, soaked in a buffer containing 5% β -mercaptoethanol for 30 min at room temperature, and placed on top of 12% slab gel for electrophoresis in the second dimension. The CTL clone 2C was analysed similarly.

encoded by any of these four additional types of γ genes. Thus at least some of the 35K material shown in Fig. 4 may be non- $V_2J_2C_2$ γ chain.

The chemical nature of the 45K component is less clear. We cannot rule out the possibility that the 'off-diagonal' 45 K component observed in Fig. 4a carries a γ epitope. If this component is a γ -chain, its relative molecular mass seems to rule out the $V_2J_2C_2$ type. On the other hand, because any one of the other four known types of γ -chains may be N -glycosylated, its molecular mass could be as large as 45K. Another possible candidate for the 45K component is the β -chain; the β gene is known to be rearranged and activated for transcription by day 16 of fetal thymocytes⁶⁻⁸. A model of intrathymic development of T cells including thymocytes bearing a $\beta\gamma$ heterodimer has been presented^{6,18,19}, but no direct evidence for such cells has been obtained to date.

Yet another possible candidate for the 45K component is a product of a new gene referred to as δ . The existence of such a polypeptide is supported by recent work on human systems. Brenner *et al.* reported the identification of a putative second T-cell receptor present on the surface of a subpopulation of peripheral blood lymphocytes (PBL) from immunodeficient patients²⁰. This protein is associated with the invariant protein CD3 (T3), and is composed of a 55K component reactive with anti- γ -peptide antibodies and a 40K polypeptide that does not react with these antibodies. Similarly Bank *et al.* reported a clone of normal immature CD4⁺8⁺ (T4⁺T8⁺) human thymocytes which bear CD3-associated dimer composed of anti- γ peptide-

reactive 44K component and anti- γ peptide non-reactive 62K component²¹. These authors propose that the component that does not react with the anti- γ peptide is a product of a new gene, δ , although its molecular mass is quite different (40K as against 62K) between the two publications.

The function of the cells bearing the putative $\gamma\delta$ heterodimer remains unknown. It has previously been reported that some CTL clones and many T helper (T_H) clones or hybridomas either lack γ RNA completely or contain only an out-of-phase γ RNA^{5,9,10,22}. These data led some to conclude that the protein encoded by the reported γ genes is not an obligatory requirement for the function of these T cells. The present study shows further that even in 2C, a CTL clone in which the γ RNA is present and in-phase, the membrane expression of the γ polypeptide chain is absent and therefore not a prerequisite of the antigen-specific function of the T cell.

One possible view of the putative $\gamma\delta$ heterodimer is that it is 'isotypic' to the conventional $\alpha\beta$ TCR, in a relationship analogous to the κ and λ light chains in immunoglobulins. Indeed the structural resemblance among α , β and γ ²³ is reminiscent of the similarity between κ and λ . Furthermore the $\gamma\delta$ -bearing cells seem to have cytotoxic²¹ or IL-2 producing capacity (D. Pardoll and A. M. Kruisbeek, personal communication). But, the apparent difference in the surface markers of the two types of T-lineage cells (CD4⁻8⁻ for $\gamma\delta$ ^{20,21} and CD4⁺8⁻ or CD4⁺8⁺ for $\alpha\beta$) does not support this view. An alternative view of the $\gamma\delta$ -bearing cells is that they define a T-cell subset other than CTL and T_H . A $\gamma\delta$ -positive human cell line exhibiting a NK (natural killer) activity seems to exist²⁴.

As the putative $\gamma\delta$ heterodimer is expressed on the surface of the fetal thymocytes, one might also envision the possibility that such a structure could function as a receptor involved in the selection of a self MHC-restricted T-cell repertoire²⁵. Before proposing a precise role of such a receptor in the expansion or deletion of specific thymocytes, however, several facts should first be established. These include the cell lineage relationship between the $\gamma\delta$ - and $\alpha\beta$ -bearing cells, the possible coexpression of the two receptors on the surface of a thymocyte subset and the functional ligands of the $\gamma\delta$ heterodimer.

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Note added in proof: Recently Lew *et al.* (*Science* **234**, 1401-1405; 1986) reported that a subpopulation of CD4⁻8⁻ adult thymocytes also express a γ -chain disulphide-bonded to a 45K component.

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1. Saito, H. *et al.* *Nature* **309**, 757-762 (1984).
2. Hayday, A. C. *et al.* *Cell* **40**, 259-269 (1985).
3. Kranz, D. M. *et al.* *Nature* **313**, 752-755 (1985).
4. Heilig, J. S. & Tonegawa, S. *Nature* **322**, 836-840 (1986).
5. Trautnecker, A., Oliveri, F., Allen, N. & Karjalainen, K. *EMBO J.* **5**, 1589-1593 (1986).
6. Raulet, D. H., Garman, R. D., Saito, H. & Tonegawa, S. *Nature* **314**, 103-107 (1985).
7. Snodgrass, H. R., Dembic, Z., Steinmetz, M. & von Boehmer, H. *Nature* **315**, 232-233 (1985).
8. Samelson, L. E. *et al.* *Nature* **315**, 765-768 (1985).
9. Reilly, E. B., Kranz, D. M., Tonegawa, S. & Eisen, H. N. *Nature* **321**, 878-880 (1986).
10. Rupp, F., Frech, G., Hengartner, H., Zinkernagel, R. M. & Joho, R. *Nature* **321**, 876-878 (1986).
11. Young, R. A. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1194-1198 (1983).
12. Kranz, D. M., Sherman, D. H., Sitkovsky, M. V., Pasternack, M. S. & Eisen, H. N., *Proc. natn. Acad. Sci. U.S.A.* **81**, 573-577 (1984).
13. Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. *Nucleic Acid Res.* **12**, 7035-7056 (1984).
14. Kreig, P. A. & Melton, D. A. *Nucleic Acid Res.* **12**, 7057-7070 (1984).
15. Goding, W. & Harris, A. W., *Proc. natn. Acad. Sci. U.S.A.* **78**, 4530-4534 (1981).
16. Garman, R. D., Doherty, P. J. & Raulet, D. H. *Cell* **45**, 733-742 (1986).
17. Iwamoto, A. *et al.* *J. exp. Med.* **163**, 1203-1212 (1986).
18. Tonegawa, S. *Sci. Am.* **253**, 122-131 (1985).
19. Pernis, B. & Axel, R. *Cell* **41**, 13-16 (1985).
20. Brenner, M. B. *et al.* *Nature* **322**, 145-149 (1986).
21. Bank, I. *et al.* *Nature* **322**, 179-181 (1986).

22. Heilig, J. S. *et al.* *Nature* **317**, 68-70 (1985).
23. Saito, H. *et al.* *Nature* **312**, 36-39 (1984).
24. Borst, J. *et al.* *Nature* **325**, 683-688 (1987).
25. Zinkernagel, R. M. *et al.* *J. exp. Med.* **147**, 882-896 (1978).
26. Mulligan, R. C. & Berg, P., *Science* **209**, 1422-1427 (1980).
27. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. *Cell* **33**, 717-728 (1983).

A γ -chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity

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We have recently derived from human fetal blood (25 wks) a series of cloned cell lines that were selected for their ability to kill the conventional natural killer (NK) target cell K562¹. It was found that a fraction of these clones express CD3 proteins but not the monomorphic Ti $\alpha\beta$ determinant recognized by WT31 antibody¹. One interleukin-2-dependent CD3⁺ WT31⁻ clone, termed F6C7, was used for immunization of mice to generate monoclonal antibodies directed at a potentially novel recognition receptor. It was shown that F6C7 cells, which transcribe Ti β but not Ti α genes, surface-express a clonotypic structure, termed NKFi². Immunoprecipitations performed with anti-NKFi monoclonal antibody (mAb) indicated that the corresponding molecule is resolved in SDS-polyacrylamide gel electrophoresis (PAGE) as a single band of relative molecular mass $\sim 85,000$ ($M_r \sim 85K$). After reduction, a major band was detected at 44K and a faint band was present at 41K². The present study was designed to characterize this structure. It was found that NKFi represents either two 44K disulphide-linked γ (TCR) chains, or possibly one γ chain associated to an additional undetected molecule, and that the 41K material corresponds to a partially glycosylated fraction of the γ protein. Anti-NKFi mAb both induces a specific autocrine proliferative response and blocks cytotoxic function, demonstrating that γ chains serve as functional receptor structures on subpopulations of normal human lymphocytes.

In a first series of experiments, we assessed whether NKFi is a glycosylated protein. After immunoprecipitation with anti-NKFi mAb, a fraction of the material was treated with endo-F, an enzyme which cleaves both high-mannose and complex glycans^{3,4}. Figure 1a depicts a typical SDS-PAGE analysis of NKFi under non-reduced conditions (lane A), reduced conditions (lane B) and reduced conditions following endo-F digestion (lane C). As shown, we usually detected two major deglycosylation products at 41K and 34K respectively. These results indicated the presence of N-linked glycans on the native 44K molecule. To establish whether the minor 41K native structure (containing generally one tenth of the total number of specific counts) was also glycosylated, each band was individually extracted from preparative gels. Separate electrophoresis of heavy (Fig. 1b, lanes A and B) and light (Fig. 1b, lanes C and D) material was then performed either directly (lane A and C) or after treatment with endo-F (lane B and D). These experiments indicated that the final digestion product of each band resolved at the same M_r of $\sim 34K$ suggesting that the

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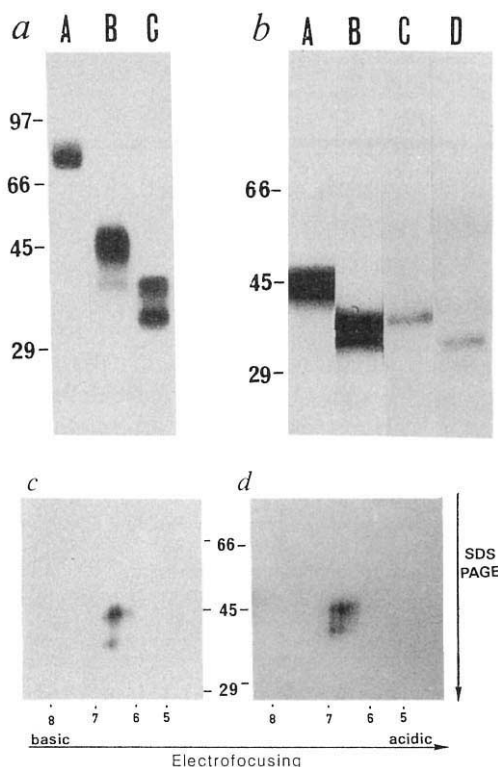


Fig. 1 Endo-F treatment of the NKFi molecule. *a*, lane A, NKFi, non-reduced conditions; lane B, NKFi reduced conditions (5% 2-mercaptoethanol (2ME)); lane C, NKFi after endo-F treatment. *b*, lane A, extracted heavy chain; lane B, heavy chain treated with endo-f; lane C, extracted light chain; lane D, extracted light chain treated with endo-F. *c*, *d*, Two-dimensional gel electrophoresis of the NKFi molecule; two representative experiments.

Methods. *a*, Specific immunoprecipitation from 125 I-labelled F6C7 cells was followed by SDS-PAGE analysis as previously reported². Endo-F treatment was performed as described³; samples were treated overnight at 37 °C with 0.2 units of endo-F (Boehringer). *b*, Heavy and light chains were cut and extracted from the gel in NH_4HCO_3 0.1 M buffer containing 0.05% SDS. After lyophilization, samples were dialysed to remove detergent and resuspended in 0.1 M Na_2HPO_4 , 0.5% SDS, 1% 2-ME, 50 mM EDTA, 1% Triton X-100, buffer pH 6.1 containing protease inhibitors, before endo-F treatment. *c*, *d*, After immunoprecipitation from 125 I-labelled F6C7 cells, the NKFi molecule was reduced with 2-mercaptoethanol and applied at the basic end of the IEF gel. The pH gradient of the first dimension was obtained with a mixture of LKB ampholines (pH 3.5–10). The gel was equilibrated in second dimension buffer and laid on the top of an SDS 10% polyacrylamide gel for protein separation according to molecular mass.

Markers shown are 29K, 45K and 66K M_r .

fainter native 41K band may well correspond to a partially glycosylated form of the major 44K structure. To study the NKFi molecule further, the immunoprecipitated material was subjected to electrofocusing before SDS-PAGE analysis. As shown in Fig. 1c, d (two representative experiments) both the heavy and light structures had similar pI values (6.5–7). But the 44K band displays a greater charge heterogeneity with more acidic material compared to the 41K band. Together these experiments favoured the hypothesis that NKFi is a unique protein species surface-expressed very largely as a disulphide-linked dimer, with a small fraction of the polypeptide being not fully glycosylated. Note that it cannot be unequivocally excluded that NKFi contains one 44K structure plus an additional molecule that is not detected under these conventional experimental procedures.

Previous studies have demonstrated the existence of 3 distinct genes $\alpha^{5,6}$, β^{7-9} and γ^{10-14} encoding either established¹⁵ or

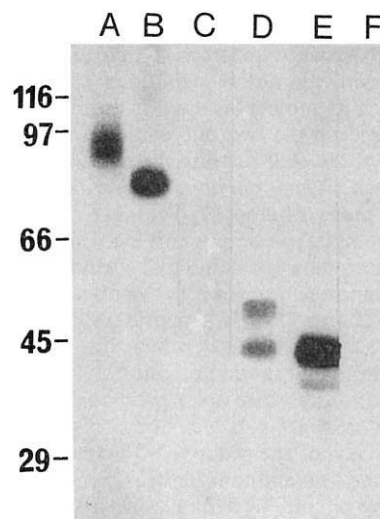


Fig. 2 Immunoprecipitations with β F1 antibody. Lanes A, D, β F1 antibody precipitations from a conventional Ti $\alpha\beta$ T-cell clone designated C15; lane B, E, anti-NKFi precipitations from F6C7 cells; lanes C, F, β F1 precipitations from F6C7 cells. SDS-PAGE was performed under either non-reduced (lanes A–C) or reduced conditions (lanes D–F).

putative¹⁶ T-cell receptor proteins. Because we could not detect Ti α transcripts in F6C7 cells, it was unlikely that the corresponding chain was part of the NKFi molecule. But a full size β message was found in the fetal lymphocytes; we therefore used anti- β F1 antibody (kindly provided by Dr M. Brenner), which is directed at a monomorphic determinant of β^{16} , to assess the potential surface expression of this protein. As shown in Fig. 2, β F1 mAb precipitated a 90K molecule from a control T-cell line under non-reduced conditions (lane A), resolving in 2 chains with respective relative molecular masses of 50K and 44K after reduction (lane D). This antibody failed to recognize any analogous structure in F6C7 cell lysates (Fig. 2, lanes C and F). To exclude the possibility that negative results simply reflect an alteration of β F1 epitope secondary to unusual association of β chains with a protein distinct from α , we performed Western blot experiments. F6C7 cell lysates were transferred to nitrocellulose membranes after electrophoresis under reduced conditions. Although specific bands were found on a variety of control T-cell lines, no signal at all was detected for F6C7 cells (not shown). We therefore conclude that β chains are not part of the NKFi molecule even though the fetal lymphocytes produce a full size β transcript which is probably not functional.

Further experiments were undertaken to investigate whether the clone type expressed on F6C7 cells contains disulphide-linked γ chains. We firstly assessed whether γ genes were transcribed in the fetal lymphocytes. As shown in Fig. 3 (lane A), a full-size 1.5-kb γ message was detected. Thus, we synthesized a 20-amino-acid peptide corresponding to a sequence (residues 117–136) encoded by the first exon of $C_{\gamma}1$ constant region^{16,17}; a series of rabbits were immunized with this peptide coupled to KLH, and specific antisera were used for immunoprecipitations. Results of these experiments were clear-cut: Fig. 4a shows that anti- C_{γ} sera precipitated from F6C7 lysates a structure resolving into two bands of 44K and 41K (Fig. 4a, lane B) which corresponds to the M_r of the protein detected with anti-NKFi under reducing conditions (Fig. 4a, lane A). Sequential immunoprecipitations were then conducted to confirm the identity between the molecule recognized by either anti- C_{γ} or anti-NKFi antibodies. It was found that pre-clearing of F6C7 lysates with anti-NKFi antibody resulted in the disappearance of the material recognized by the C_{γ} antisera

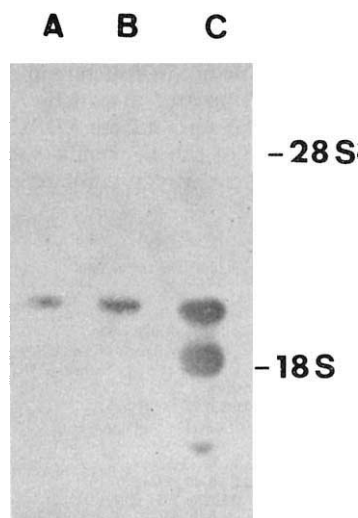


Fig. 3 Northern blot analysis of F6C7 cloned cell line with γ probe. Lane A, F6C7 cells; lane B, pH 28 cells, control T-cell clone known to transcribe γ chains (without apparent surface expression); lane C, JM cells, T cell leukaemia.

Methods. RNA was prepared from the cytoplasmic fraction of cells lysed in hypotonic buffer in the presence of Nonidet-P40. Total RNA was purified by two phenol-chloroform extractions, followed by two ethanol precipitations. The RNA preparations (5 μ g per slot) were dissolved with water, heated at 50 °C for 1 h in the presence of 1 M glyoxal, 50% DMSO and run in a 1% agarose gel at 30 V for 5 h. The gel was soaked in 10 $\times$ SSC for 30 min and the RNA transferred on two Genescreen Plus nylon membranes placed on both sides of the gel. Hybridizations were carried out in 50% formamide, 5 $\times$ SSC, 1 $\times$ Denhardt's, 5% dextran sulphate with 1 $\times$ 10⁷ counts per minute (c.p.m.) ml⁻¹ of ³²P-labelled RNA C γ probe at 65 °C for 16 h. This high specific activity probe was synthesized with T3 RNA polymerase and ³²P-UTP (800 Ci mmol⁻¹) using as template a BamHI-AccI C γ fragment isolated from a PH28 cDNA library established in λ gt10. The nylon membranes were washed twice in 2 $\times$ SSC, twice in 2 $\times$ SSC 1% SDS at 60 °C for 30 min and twice in 0.1 $\times$ SSC, 0.1% SDS at 65 °C for 30 min.

(Fig. 4a, lane C). (It was not possible to perform the reverse experiments because anti-C γ sera did not precipitate the native molecule and specific binding of anti-NKFi antibody was abrogated by the denaturation procedure.) To confirm that both the 44K and 41K bands contain the same protein species, they were individually extracted from gels and subsequently subjected to anti-C γ immunoprecipitations. As shown in Fig. 4b (lane B and E), both structures were individually reprecipitated by the specific heteroantisera.

Together our results support the view that NKFi represents a disulphide-linked γ - γ homodimer although, as already mentioned, we cannot exclude the possibility that a γ -associated protein has not been detected. In any case, it appears most likely

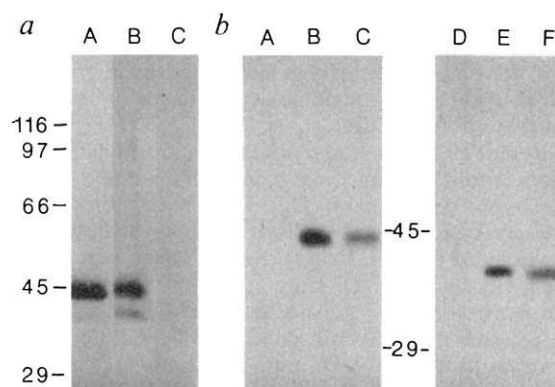


Fig. 4 a, Immunoprecipitating with C γ antisera. Lane A, labelled F6C7 lysate immunoprecipitated with anti-NKFi antibody. SDS-PAGE was performed in the presence of 5% 2-mercaptoethanol. Lane B, immunoprecipitation with anti C γ antisera following denaturation-renaturation procedures which include DTT treatment of the lysate¹⁶. Lane C, immunoprecipitation with anti-C γ antisera under conditions described in lane B but after preclearing the lysates twice with anti-NKFi. b, Precipitation of both 44K and 41K NKFi chains. Lane A, heavy chain precipitated with rabbit preimmune sera; lane B, heavy chain precipitated with C γ antisera; lane C, heavy chain; lane D, light chain precipitated with rabbit preimmune sera; lane E, light chain precipitated with C γ antisera; lane F, light chain.

Methods. a, A 20-amino-acid peptide (residues 117-136) from the constant region of the γ chain^{16,17} was synthesized using an Applied Biosystems apparatus according to the Merrifield method¹⁸. Following quality control by amino-acid analysis (LKB amino-acid analyser alpha) and fast atom bombardment mass spectrometry (VG400 mass spectrometer), the peptide was coupled to keyhole limpet haemocyanin (KLH). Various antisera were screened for specific reactivity with the immunizing peptide using appropriate ELISA assays; sera selected for further studies had specific reactivity in this experimental system at 1/10,000 dilution. Immunoprecipitations with γ -specific antisera were performed on F6C7 cell lysates using protein A-Sepharose beads after denaturation with SDS (final concentration 1%) reduction with DTT (2 mM) and subsequent heating (4 min at 100 °C) before addition of iodoacetamide (20 mM final concentration) as described by Brenner *et al.*¹⁶. b, Material precipitated from F6C7 cells with anti-NKFi antibody was subjected to SDS-PAGE and heavy and light chains were cut and extracted separately from the gel as described in Fig. 1. This material was prepared for immunoprecipitation as above and submitted to subsequent treatment with either anti-C γ antisera (lanes B, E) or control preimmune sera (lanes A, D). Part of the extracted material was run in parallel as a control for separation of heavy (lane C) and light (lane F) chains. Note that the same amount of radioactivity was analysed in lanes B and C, or lanes E and F respectively.

that diversity exists in surface expression of γ chains. Indeed, the presence of this protein has been recently demonstrated on circulating lymphocytes from immunodeficient patients¹⁶ as well as immature thymocytes¹⁹. But the CD3-associated material described by Brenner *et al.* was found to consist of two non-

Table 1 Proliferative response of F6C7 cells following stimulation by anti-T3 or anti-NKFi antibody

	Medium	rIL2	Anti-T3	Anti-T3 + rIL2	Anti-NKFi	Anti-NKFi + rIL2
F6C7	1,992 ±190	23,181 ±3,282	17,145 ±917	42,660 ±1,749	56,429 ±4,972	45,059 ±2,636
PH.28	1,579 ±46	31,260 ±2,639	32,689 ±6,206	80,313 ±4,751	2,222 ±102	39,157 ±1,015

Results represent the mean of ³H-thymidine uptake in c.p.m. ± s.d. from triplicate cultures. 6 $\times$ 10⁴ cells per well were cultured in round-bottom plates for 3 d, including a 16-h pulse in the presence of either medium or antibodies coupled to BrCN Sepharose beads or rIL2 (final concentration 50 U ml⁻¹) or both. PH.28 is a CD3⁺ CD4⁺ inducer control clone specific for diphtheria toxoid. Note that anti-NKFi induces a strong proliferation without the addition of exogenous interleukin-2. This proliferation was not enhanced if interleukin-2 was added in the experimental system demonstrating that the specific response developed through an autocrine pathway.

disulphide-linked molecules including γ and a putative additional structure designated δ^{16} . Thus, future experiments will have to dissect heterogeneity in membrane organization of these novel receptors and elucidate its biological relevance in T-lymphocyte differentiation.

As described in this study, NKFi structure is defined by both constant region antisera and a monoclonal antibody specific for a clonotypic determinant. This latter reagent recognizes the native molecule on viable cells and has been shown² to block cytotoxicity of the F6C7 clone. Furthermore, experiments performed here (Table 1) show that anti-NKFi coupled to Sepharose beads induces a specific signal for autocrine proliferation, which is probably transduced through CD3 proteins given the similar effect of anti-CD3 antibody (Table 1). It can therefore be concluded that γ chains actually serve as functional receptor structures on subpopulations of human lymphocytes.

The unique CD3⁺ WT31⁻ phenotype which appears to be predictive of γ chain surface expression was originally described¹ in studying human fetal lymphocytes selected for their ability to display 'natural killer-like' function. We have now generated a number of CD3⁺ cloned cell lines derived from either thymus or adult normal peripheral blood which were characterized because they do not react with WT31 monoclonal antibody. Each of these later IL-2-dependent CD3⁺ WT31⁻ clones kill genetically unrelated tumour cell lines. Thus, further experiments are necessary to confirm whether surface expression

of γ chains consistently correlates with such a non-MHC restricted cytotoxic function and, perhaps more importantly, to establish the potential role of this structure in target cell recognition.

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- Nowill, A. *et al.* *J. exp. Med.* **163**, 1601-1606 (1986).
- Moingeon, P. *et al.* *Nature* **323**, 638-640 (1986).
- Elder, J. H. & Alexander, S. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4540-4544 (1982).
- Plummer, T. H., Elder, J. H., Alexander, S., Phelan, A. W. & Tarentino, A. L. *J. biol. Chem.* **259**, 10700-10704 (1984).
- Yanagi, Y. *et al.* *Nature* **308**, 145-149 (1984).
- Hedrick, S., Nielsen, E. A., Kavalier, S., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153-158 (1984).
- Chien, Y. *et al.* *Nature* **312**, 31-35 (1984).
- Saito, H. *et al.* *Nature* **312**, 36-40 (1984).
- Sim, G. K. *et al.* *Nature* **312**, 771-775 (1984).
- Lefranc, M. P. & Rabbitts, T. H. *Nature* **316**, 464-466 (1985).
- Saito, H. *et al.* *Nature* **309**, 757-762 (1984).
- Kranz, D. M. *et al.* *Nature* **313**, 752-755 (1985).
- Hayday, A. C. *et al.* *Cell* **40**, 259-269 (1985).
- Murre, C. *et al.* *Nature* **316**, 549-552 (1985).
- Meuer, S. C. *et al.* *Nature* **303**, 808-810 (1983).
- Brenner, M. B. *et al.* *Nature* **322**, 145-149 (1986).
- Dialynas, D. P. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **83**, 2619-2623 (1986).
- Merrifield, R. B. *et al.* *J. Am. chem. Soc.* **85**, 2149-2154 (1963).
- Bank, I. *et al.* *Nature* **322**, 179-181 (1986).

Independent phosphatidylinositol synthesis in pituitary plasma membrane and endoplasmic reticulum

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Phosphatidylinositol (PtdIns), the most abundant phosphoinositide, is the precursor of phosphatidylinositol 4-monophosphate which is converted to phosphatidylinositol 4,5-bisphosphate, the lipid hydrolysed as an early step in signal transduction by many stimuli¹⁻³. It is generally thought that a single enzyme in the endoplasmic reticulum, PtdIns synthase (CDP-diglyceride:myo-inositol 3-phosphatidyltransferase, EC 2.7.8.11), is responsible for PtdIns synthesis and that newly synthesized PtdIns is transported to the plasma membrane by exchange proteins⁴. Several investigators have proposed that there are two functionally distinct pools of PtdIns, one responsive to stimulation and the other not⁵⁻⁸, and that the stimulus-responsive pool may be synthesized at a different site within the cell, perhaps within the plasma membrane. Indeed, it was suggested that there is PtdIns synthase activity in plasma membrane isolated from rat liver⁹. GH₃ rat pituitary tumour cells are an excellent model system to study stimulation of phosphoinositide metabolism by thyrotropin-releasing hormone (TRH)¹⁰. Conversion of PtdIns to polyphosphoinositides¹¹ and TRH (and GTP)-activated phosphoinositide hydrolysis^{12,13} are known to occur in plasma membrane isolated from GH₃ cells. Here we report that PtdIns synthase activity in the plasma membrane of GH₃ cells is distinct from that present in the endoplasmic reticulum. The plasma membrane PtdIns synthase may be responsible for a portion of PtdIns re-synthesis that occurs during cell stimulation.

Figure 1 illustrates the profile of activity of myo-[³H]inositol incorporation into PtdIns in GH₃ cell membranes fractionated on a continuous sucrose density gradient. There are two peaks

of activity, corresponding by marker enzyme analysis to the plasma membrane and endoplasmic reticulum fractions. More than 98% of the ³H-radioactivity incorporated into lipid was in PtdIns, which was membrane-bound as it was recovered in the pellet after centrifugation at 100,000g for 60 min. Using the standard assay buffer, the specific activity in the plasma membrane was approximately twice that in the endoplasmic reticulum. In contrast, as reported in other cell types^{9,14}, activity in the endoplasmic reticulum causing incorporation of myo-inositol into PtdIns could be increased several-fold above that in plasma membrane by using different assay conditions, for example by increasing the concentration of Mn²⁺ or substrates (Fig. 2c). Nevertheless, because the total protein recovered in the two fractions was the same (29 ± 5.4 versus 30 ± 2.6 µg per 10⁷ cells), the total activity that causes myo-inositol incorporation into PtdIns, when assayed in a buffer without Mn²⁺ or in a buffer that more closely simulates cytosolic conditions (Fig. 2 legend), was greater in plasma membrane than in endoplasmic reticulum.

In addition to PtdIns synthase, enzyme-mediated base exchange, which does not lead to net synthesis of PtdIns and can occur in the absence of CDP-diglyceride, can be responsible for incorporation of myo-[³H]inositol into PtdIns¹⁵. The contribution of base exchange, incorporation in the absence of added CDP-diglyceride, to the activity measured in plasma membrane and endoplasmic reticulum was 21 ± 9% and 1.6 ± 0.6%, respectively (Fig. 2a); these may be overestimations because endogenous CDP-diglyceride may have been present in the membrane fractions. Evidence that supports the notion that myo-[³H]inositol incorporation in the absence of CDP-diglyceride was by base exchange and in the presence of CDP-diglyceride was mainly by *de novo* synthesis catalysed by PtdIns synthase, was obtained in experiments in which GH₃ cell membranes were incubated with myo-[³H]inositol without or with CDP-diglyceride and then a large excess of unlabelled myo-inositol was added. The myo-[³H]inositol incorporated into PtdIns in the absence of CDP-diglyceride was readily chased by unlabelled myo-inositol, whereas that incorporated in the presence of CDP-diglyceride was not affected during subsequent incubation with unlabelled myo-inositol (data not shown).

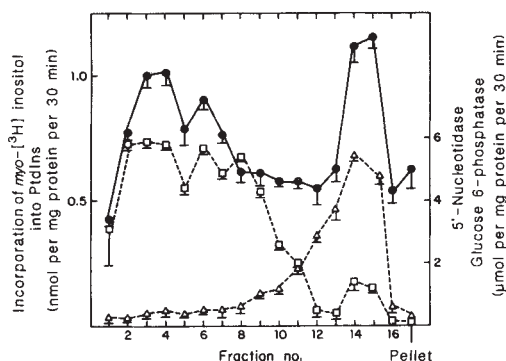


Fig. 1 Distribution of PtdIns synthase (●), 5'-nucleotidase (□) and glucose 6-phosphatase (△) specific activities in membranes isolated from GH₃ cells after centrifugation on a continuous sucrose density gradient.

Methods. GH₃ cells were grown as monolayer cultures in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37 °C and collected with 0.02% EDTA²⁴. Lysis buffer (0.5 mM dithiothreitol, 1 mM EGTA, 0.5 mM sodium bicarbonate and 10 mM HEPES, pH 7.9) was added (~4 to 5 times the cell pellet volume) and the cells were suspended and simultaneously lysed by incubating at 37 °C for 4–5 min, followed by addition of 10 volumes of ice-cold lysis buffer and rapid mixing on a vortex mixer for 0.5 min at 0–4 °C. The homogenate was centrifuged at 800g for 10 min to eliminate nuclei and cell debris. The supernatant was centrifuged again at 100,000g for 1 h and the pellet was layered on top of a continuous sucrose density gradient, 25–50% (w/v), and then centrifuged at 100,000g in a swinging-bucket rotor for 1 h. The fractions were washed with lysis buffer and assayed immediately. PtdIns synthase activity was measured as the incorporation of *myo*-[³H]inositol into PtdIns in the presence of CDP-diglyceride. The assay buffer contained 100 mM Tris-HCl, pH 7.5, 1 mM EGTA, 3 mM MgCl₂, 3 mM MnCl₂, 0.1 mM CDP-dipalmitoyl glycerol (Sigma), 0.1 mM *myo*-[³H]inositol (~1 × 10⁵ d.p.m. nmol⁻¹, NEN) and membrane protein (20–50 µg) in a total volume of 0.05 ml. CDP-diglyceride was added after dispersal by brief sonication. Similar results were obtained when the activities were measured in buffer containing 100 mM KCl and 10 or 20 mM NaCl in the presence or absence of Mn²⁺. The reaction was started by the addition of membrane suspensions, and was incubated at 37 °C for 30 min. Under these conditions, the rate of formation of PtdIns was constant for at least 60 min. The reactions were terminated by adding 1 ml of chloroform/methanol/concentrated HCl (100:100:1, v/v/v) and the phases separated with 0.25 ml of 10 mM EDTA. The upper phase was re-extracted with pre-equilibrated lower phase, and combined with the lower phases. The extracted lipid was washed with 1 ml pre-equilibrated upper phase. PtdIns was isolated by thin-layer chromatography on silica gel 60 using a solvent system of chloroform/methanol/acetic acid/water (50:30:8:4, v/v/v/v). Most (>98%) of the radioactivity in the lipid fraction co-migrated with authentic PtdIns. Marker enzyme analyses were performed using 5'-nucleotidase²⁵ and (Na⁺-K⁺)ATPase²⁷ as markers for plasma membrane, succinate dehydrogenase²⁸ for mitochondria, and glucose 6-phosphatase²⁹ and NADH dehydrogenase³⁰ for endoplasmic reticulum; only 5'-nucleotidase and glucose 6-phosphatase are shown. Although not specifically assayed, it is probable that the plasma membrane fractions were contaminated with membranes from Golgi apparatus. Protein was determined according to the method of Lowry *et al.*³¹ using bovine serum albumin (BSA) as standard.

Hence, most of the activity that caused incorporation of *myo*-[³H]inositol into PtdIns measured in plasma membranes and endoplasmic reticulum was caused by PtdIns synthase.

The PtdIns synthase activity in plasma membrane is different from the activity in endoplasmic reticulum (Fig. 2). The apparent *K_m* values for CDP-diglyceride and *myo*-inositol, calculated from double reciprocal plots, were different. For *myo*-inositol, *K_m* was 0.067 ± 0.009 and 0.26 ± 0.045 mM (*P* < 0.001) for plasma membrane and endoplasmic reticulum activities, respectively, and for CDP-diglyceride, *K_m* was 0.040 ± 0.016 and 0.33 ± 0.12 mM (*P* < 0.01) for plasma membrane and endoplasmic reticulum activities, respectively. A more marked difference was found in the effect of Mn²⁺ in enhancing PtdIns synthase activities in plasma membrane and endoplasmic reticulum. For Mn²⁺, *K_m* was 0.015 ± 0.005 and 3.3 ± 1.3 mM (*P* < 0.005) for plasma membrane and endoplasmic reticulum, respectively. In contrast, the effect of Mg²⁺ in stimulating PtdIns synthase activity (half-maximal stimulation at 0.03–0.1 mM Mg²⁺), of pH (optimum ~7.5), and of Ca²⁺ in inhibiting activity (half-maximal inhibition at 0.1 mM free Ca²⁺ and maximal inhibition of >80%

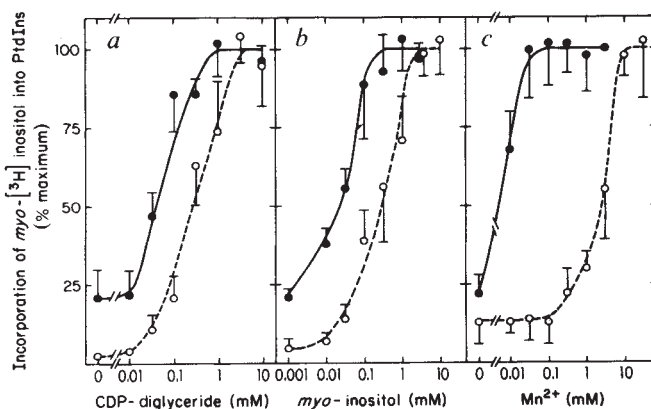


Fig. 2 Effects of *a*, *myo*-inositol; *b*, CDP-diglyceride; and *c*, Mn²⁺ on the incorporation of *myo*-[³H]inositol into PtdIns in plasma membrane (●) and endoplasmic reticulum (○) isolated from GH₃ cells.

Methods. GH₃ cells were grown, collected and lysed, and a post-nuclear fraction obtained, as in Fig. 1. The resulting pellet was resuspended in lysis buffer that was layered on a discontinuous sucrose gradient consisting of 30, 36, 41, 45 and 50% (w/v) sucrose in lysis buffer. The gradient was centrifuged at 100,000g for 1 h yielding five narrow bands at the interfaces that were collected separately. The upper and lower two bands were collected as the plasma membrane and endoplasmic reticulum fractions, respectively. Incorporation of *myo*-inositol into PtdIns in the plasma membrane or endoplasmic reticulum fractions was measured as in Fig. 1, except that the concentration of CDP-diglyceride, *myo*-[³H]inositol, or MnCl₂ was varied.

at 1 mM free Ca²⁺) were similar. These data demonstrate that PtdIns synthase activities in plasma membrane and endoplasmic reticulum are distinct; they do not show that there are two PtdIns synthases because the same enzyme acting in different environments could display different properties.

Phosphoinositide kinases are present within plasma membranes^{11,16} and recent experiments using membranes isolated from several cell types^{17,18}, including GH₃ cells^{12,13}, have demonstrated stimulus-induced, phospholipase-C-mediated phosphoinositide hydrolysis, which is dependent on guanine nucleotides¹⁹. These findings confirm the suggestion that at least part of phosphoinositide degradation in intact cells occurs in the plasma membrane. However, experiments performed to show phosphoinositide degradation²⁰ or re-synthesis²¹ in plasma membrane after stimulation of intact cells have been inconclusive. We attempted to discover the location of enhanced re-synthesis of PtdIns in intact GH₃ cells during TRH stimulation by monitoring incorporation of *myo*-[³H]inositol. When intact cells were incubated in buffer containing micromolar to millimolar concentrations of *myo*-[³H]inositol for 10–45 min in the presence or absence of TRH, we were unable to show any increase in incorporation into plasma membrane (data not shown). Because the loss of unesterified *myo*-inositol from cells was slow (unpublished data), we considered the possibility that the inability to measure stimulated incorporation of *myo*-[³H]inositol was caused by insufficient depletion of endogenous *myo*-inositol. We therefore attempted to deplete cellular *myo*-inositol by transiently permeabilizing the cells in *myo*-inositol-free buffer and then resealing them in the presence of *myo*-[³H]inositol; cells made permeable and then resealed are responsive to TRH²². In resealed cells incubated with *myo*-[³H]inositol, specific ³H-radioactivity in PtdIns was similar in plasma membrane and in endoplasmic reticulum (Table 1). TRH caused 2.4- and 2.2-fold increases in specific ³H-radioactivity in plasma membrane PtdIns and endoplasmic reticulum PtdIns, respectively. Because the plasma membrane content of PtdIns was similar to that of the endoplasmic reticulum, the rapid and similar increases in specific ³H-radioactivity in PtdIns stimulated by TRH in both membrane fractions make it unlikely that most

Table 1 Effect of TRH on the specific ^3H -radioactivity in PtdIns in plasma membrane and endoplasmic reticulum isolated from permeabilized and resealed GH₃ cells

	Specific ^3H -radioactivity in PtdIns (d.p.m. nmol ⁻¹)	
	Control	TRH
Plasma membrane	887 ± 13	2,110 ± 156
Endoplasmic reticulum	883 ± 103	1,920 ± 241

Cells were permeabilized and resealed by the method of Snowdowne and Borle²². In brief, cells were collected and washed as described in Fig. 1 legend and resuspended in 2 ml hypotonic buffer containing 3 mM HEPES, pH 7.4, and 3 mM ATP at 4 °C. After 3 min, 1 μM myo-[^3H]inositol was added followed by 0.14 ml of a solution containing 2 M KCl and 3 mM HEPES. After 15 min, the cells were washed and resuspended in a buffer consisting of 135 mM NaCl, 4.5 mM KCl, 0.5 mM MgCl₂, 1.5 mM CaCl₂, 5.6 mM glucose and 10 mM HEPES, pH 7.4 at 37 °C. After an additional 15 min, cells were incubated without (control) or with 1 μM TRH for 5 min. The membrane fractions were isolated as described in Fig. 1 legend and the incorporation of ^3H -radioactivity and the phosphorus content of PtdIns were determined²⁴. PtdIns was isolated by two-dimensional thin-layer chromatography²⁶. The subcellular distribution of PtdIns, measured as stable phosphorus²⁴, was 39 ± 6.6% in plasma membranes, 25 ± 6.1% in the endoplasmic reticulum and 36 ± 9.0% in mitochondria.

plasma membrane PtdIns is synthesized in the endoplasmic reticulum and then transported to the plasma membrane.

These data demonstrate that PtdIns is synthesized within plasma membrane as well as endoplasmic reticulum of GH₃ cells and provide initial evidence that during stimulation of intact cells by TRH both sites are active. Although our data were derived from experiments that used a rat pituitary tumour cell line, our findings may be applicable to all cells that respond to stimuli that use phosphoinositide hydrolysis as an early step in signal transduction. The findings reported here are important in understanding phosphoinositide metabolism during cell stimulation by showing that rapid, vectorial flow of newly synthesized PtdIns from the endoplasmic reticulum to a stimulus-responsive phosphoinositide pool⁵⁻⁸ is not needed. Because recent findings that stimulation of PtdIns hydrolysis is persistent whereas that of phosphatidylinositol 4,5-bisphosphate is transient²³⁻²⁵ are compatible with a direct role for PtdIns hydrolysis in the prolonged actions of stimuli, it is possible that hydrolysis of PtdIns within the plasma membrane is the mechanism of prolonged generation of diacylglycerol for activation of protein kinase C. Indeed, our observations suggest that a stimulus-responsive pool of phosphoinositides is present within the plasma membrane and that both hydrolysis and re-synthesis of this pool of phosphoinositides occurs in it.

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1. Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. *Phil. Trans. R. Soc. B* **296**, 123-137 (1981).
2. Nishizuka, Y. *Nature* **308**, 693-698 (1984).
3. Berridge, M. J. & Irvine, R. F. *Nature* **312**, 315-321 (1984).
4. Bell, R. M. A. *Rev. Biochem.* **49**, 459-487 (1980).
5. Fain, J. N. & Berridge, M. J. *Biochem. J.* **180**, 655-661 (1979).
6. Monaco, M. E. & Woods, D. J. *biol. Chem.* **258**, 15125-15129 (1983).
7. Rana, R. S. J. *biol. Chem.* **260**, 7861-7867 (1985).
8. Koreh, K. & Monaco, M. E. *J. biol. Chem.* **261**, 88-91 (1986).
9. Takenawa, T., Saito, M., Nagai, Y. & Egawa, K. *Archs Biochem. Biophys.* **182**, 244-250 (1977).
10. Gershengorn, M. C. A. *Rev. Physiol.* **48**, 515-526 (1986).
11. Imai, A., Rebecchi, M. J. & Gershengorn, M. C. *Biochem. J.* **240**, 341-348 (1986).
12. Straub, R. E. & Gershengorn, M. C. *J. biol. Chem.* **261**, 2712-2717 (1986).
13. Martin, T. F. J., Lucas, D. O., Bajjalieh, S. M. & Kowalchuk, J. A. *J. biol. Chem.* **261**, 2918-2927 (1986).
14. Paulus, H. & Kennedy, E. P. *J. biol. Chem.* **235**, 1303-1311 (1960).
15. Bleasdale, J. E. & Wallis, P. *Biochim. biophys. Acta* **664**, 428-440 (1981).
16. Irvine, R. F. *Cell Calcium* **3**, 295-309 (1982).
17. Litosch, I., White, C. & Fain, J. N. *J. biol. Chem.* **260**, 5464-5471 (1985).
18. Smith, C. D., Lane, B. C., Kusaka, I., Verghese, M. W. & Snyderman, R. *J. biol. Chem.* **260**, 5875-5878 (1985).
19. Cockcroft, S. & Gomperts, B. D. *Nature* **314**, 534-536 (1985).
20. Bennett, J. P., Cockcroft, S., Caswell, A. H. & Gomperts, B. D. *Biochem. J.* **208**, 801-808 (1982).
21. Seyfred, M. A. & Wells, W. W. *J. biol. Chem.* **259**, 7666-7672 (1984).
22. Snowdowne, K. W. & Borle, A. B. *Am. J. Physiol.* **246**, E198-E201 (1984).
23. Wilson, D. B., Newfeld, E. J. & Majerus, P. W. *J. biol. Chem.* **260**, 1046-1051 (1985).

24. Griendling, K. K. *et al. J. biol. Chem.* **261**, 5901-5906 (1986).
25. Imai, A. & Gershengorn, M. C. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8540-8544 (1986).
26. Rebecchi, M. J., Kolesnick, R. N. & Gershengorn, M. C. *J. biol. Chem.* **258**, 227-234 (1984).
27. Evans, W. H. in *Laboratory Techniques in Biochemistry and Molecular Biology* Vol. 7 (eds Work, T. S. & Work, E.) 103-105 (Elsevier, New York, 1980).
28. Evans, W. H. in *Laboratory Techniques in Biochemistry and Molecular Biology* Vol. 7 (eds Work, T. S. & Work, E.) 107-108 (Elsevier, New York, 1980).
29. Bonner, W. D. *Meth. Enzym.* **1**, 723-729 (1955).
30. Evans, W. H. in *Laboratory Techniques in Biochemistry and Molecular Biology* Vol. 7 (eds Work, T. S. & Work, E.) 115 (Elsevier, New York, 1980).
31. Galante, Y. M. & Hatefi, Y. *Meth. Enzym.* **53**, 15-21 (1978).
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. L. *J. biol. Chem.* **193**, 265-275 (1951).
33. Imai, A., Ishizuka, Y., Nakashima, S. & Nozawa, Y. *Archs. Biophys. Biochem.* **232**, 259-268 (1984).

Coevolution of codon usage and transfer RNA abundance

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The use of synonymous codons is strongly biased in the bacterium *Escherichia coli* and yeast, comprising both bias between codons recognized by the same transfer RNA and bias between groups of codons recognized by different synonymous tRNAs (reviewed in ref. 1). A major determinant of the second sort of bias is tRNA content, codons recognized by abundant tRNAs being used more often than those recognised by rare tRNAs, particularly in highly expressed genes, probably owing to selection at the level of translation against codons recognized by rare tRNAs¹⁻³. Conversely, codon usage is likely to exert selection pressure on tRNA abundance⁴. Here I develop a model for the coevolution of codon usage and tRNA abundance which explains why there are unequal abundances of synonymous tRNAs leading to biased usage between groups of codons recognized by them in unicellular organisms.

Consider first the evolution of codon usage with tRNA abundance held constant. At a particular coding position suppose that there are two groups of synonymous codons, A₁ and A₂, recognized by two isoaccepting tRNAs. Suppose that the second tRNA is more rare than the first so that A₂ has relative fitness 1 - s compared with A₁ (s > 0), and let u be the mutation rate between A₁ and A₂, assumed the same in each direction. Because s is likely to be very small, the relative frequencies of A₁ and A₂, denoted by p and q = 1 - p, will be determined by mutation-selection balance. Ignoring non-synonymous mutations and drift, the equilibrium frequency of A₂ is

$$q = \frac{1}{2} + \frac{u}{s} - \frac{1}{2} \left[4 \left(\frac{u}{s} \right)^2 + 1 \right]^{1/2} \quad (1)$$

(If A₂ is at a selective advantage so that s < 0, the sign of the last term becomes positive.) This model, assuming independent selection at each coding position, seems more plausible⁵ than Kimura's model⁴ which supposes that there is an optimum frequency of A₁ codons in the genome.

Availability of tRNA affects translation time⁶⁻⁹ and it has been suggested⁶ that the time, t, taken to translate a codon can be expressed as

$$t = \theta / C + \tau \quad (2)$$

where θ is the mean waiting time before the arrival of a tRNA plus the time taken to test it, C is the relative abundance of the matching tRNA (scaled to sum to unity, so that 1/C is the average number of tRNAs tested before a match is found), and τ is the time taken for transpeptidation and translocation (likely to be small compared with the first term⁶⁻¹⁰). Other factors affecting codon usage, such as contextual constraints¹¹ and mRNA secondary structure are ignored in equation (2), on the grounds that they are of minor importance compared with tRNA

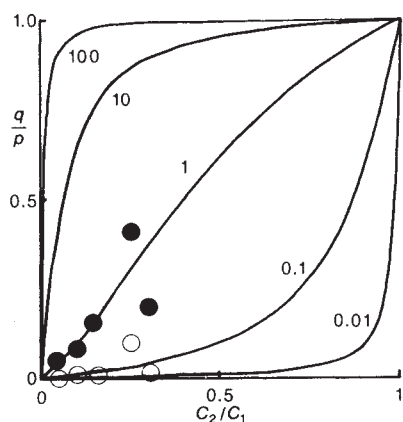


Fig. 1 Predicted codon usage (q/p) plotted against amount of tRNA (C_2/C_1) at different levels of gene expression (inversely proportional to u/k , shown on each curve) when codon usage evolves at fixed tRNA abundance to an equilibrium mutation-selection balance. Points are observed data¹ in *E. coli* for the strongly expressed gene *ompA* (○) and the weakly expressed *lac* repressor gene (●).

abundance⁶ and will average out when comparison is made between groups of codons recognized by different tRNAs.

An increase in translation time may reduce fitness either directly, because rapidity of clonal division is a component of fitness, or indirectly through the extra energy needed to test and reject non-cognate tRNAs. In either case, if t_1 and t_2 are the average times taken to translate codons in groups A_1 and A_2 , we may suppose that

$$s = \alpha(t_2 - t_1) = k \left(\frac{C_1}{C_2} - 1 \right) \quad (3a)$$

$$k = \alpha \theta / C_1 \quad (3b)$$

where α depends on the degree of expression of the gene. Because C_1 , the relative abundance of the major tRNA, is about the same for all amino acids¹, k can be regarded as a constant within a gene and equations (1) and (3) can be used to calculate q/p as a function of C_2/C_1 given the single parameter u/k . Theoretical curves for different values of u/k are shown in Fig. 1. If the forward and backward mutation rates are unequal, say u_1 from A_1 to A_2 and u_2 the other way, similar curves would be obtained starting at the origin but meeting at $q/p = u_1/u_2$ when $C_2/C_1 = 1$.

Ikemura¹ has plotted tRNA usage against amount of tRNA for different genes of *E. coli* and yeast; for each amino acid both variables were normalized to 1 for the most abundant tRNA. These variables are directly comparable with the theoretical variables plotted in Fig. 1, and the observed data are shown for comparison for a weakly expressed gene (the *lac* repressor, *lacI*) and a highly expressed gene (the outer membrane protein gene, *ompA*) in *E. coli*. The results for *lacI* lie approximately on the curve predicted for $u/k = 1$, whereas those for *ompA* lie on one of the markedly convex curves (indicating a much stronger bias) predicted when $u/k \ll 1$, consistent with the expectation that k increases with the degree of gene expression.

It is of interest to see whether a very simple model relating fitness to translation time can generate a selection pressure of the right order of magnitude. Suppose that the i th codon in the genome takes t_i seconds to translate and that this codon is used r_i times during one mitotic division, so that r_i molecules of its gene product are made. Under optimal growth conditions, the time taken for a division is $T = \sum r_i t_i / R$, where R is the number of ribosomes. Under these conditions, the fitness of an organism (W) can be taken as the reciprocal of the division time, $W = 1/T$. Hence for a codon used r times, $\alpha = r/RT$ (see equation (3)) and $k = r\theta/C_1 RT$. For *E. coli*, doubling twice an hour¹⁰, $\theta/C_1 \approx$ translation time per codon = 0.061 s, $R = 9,400$, so that $k =$

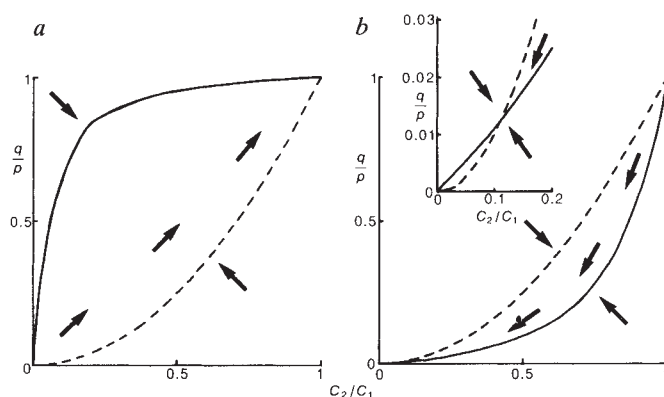


Fig. 2 Predicted codon usage (q/p) plotted against amount of tRNA (C_2/C_1) for $u/k = (a) 10$, $(b) 0.1$. Solid curve shows evolution of codon usage with fixed tRNA abundance (see Fig. 1). Dashed curve shows evolution of tRNA abundance with codon usage fixed. Inset in (b) is an enlargement near the origin. Arrows show the direction of movement, which has an upward or downward component according to whether the point is below or above the solid curve, and a left or right component according as it is to the right or left of the dashed curve.

$3.6r \times 10^{-9}$. Estimates of r are 40 and 3×10^4 for *lacI*³ and *ompA*¹, giving $k \approx 10^{-7}$ and 10^{-4} respectively. For *lacI*, Fig. 1 suggests that $k \approx u$, the mutation rate per site, which is probably less than 10^{-7} . Thus the model has overestimated the selection pressure, which is perhaps not surprising in view of its crudity, but the calculation shows that the direct effect of translation time on fitness in a clonal organism can generate enough selection to account for the bias in codon usage observed in Fig. 1.

Now consider the evolution of tRNA abundance with codon usage held fixed. Thus, suppose that q is fixed but that C_2 can evolve, with $C_1 + C_2$ held constant; this constraint is appropriate as we want to determine the optimal allocation of resources between the two tRNAs. Consider a mutant which increases C_2 to $C_2 + d$ and decreases C_1 to $C_1 - d$, where d is small. From equation (2) the change in the translation time is $\Delta t_1 = \theta d / C_1^2$ for an A_1 codon and $\Delta t_2 = -\theta d / C_2^2$ for an A_2 codon. From equation (3) the selection coefficient against the mutant is

$$\Delta t_1 \sum_1 \alpha + \Delta t_2 \sum_2 \alpha = d C_1 \left(C_1^{-2} \sum_1 k - C_2^{-2} \sum_2 k \right) \quad (4)$$

where $\sum_1$ and $\sum_2$ denote summation over A_1 and A_2 codons respectively. Setting (4) equal to zero at equilibrium, $(C_2/C_1)^2 = \sum_2 k / \sum_1 k$.

To take into account differences in codon usage and in the selection intensity k between poorly and highly expressed genes, divide genes into groups with different levels of expression and suppose that a fraction π_j of the codons belong to the j th group and that in this group $k = k_j$ and the relative frequencies of A_1 and A_2 are p_j and q_j respectively. Then $\sum_2 k / \sum_1 k = \bar{q} / \bar{p}$ where $\bar{p}$ and $\bar{q}$ are the weighted averages $\bar{p} = \sum_j w_j p_j$, $\bar{q} = \sum_j w_j q_j$ with $w_j = \pi_j k_j / \sum_j \pi_j k_j$. Hence

$$(C_2/C_1)^2 = \bar{q} / \bar{p} \quad (5)$$

This result depends on the assumption that selection pressure is inversely proportional to tRNA abundance.

We can now investigate the coevolution of codon usage and tRNA abundance by combining the separate analyses. Suppose first that all genes have the same degree of expression. Figure 2 shows the superposition of the curve for the evolution of codon usage (solid, reproduced from Fig. 1) and the curve for the evolution of tRNA abundance (dashed, from equation (5)). Two values of u/k , representing different levels of gene expression, are illustrated. In each case the equilibria under coevolution are the points of intersection of these two curves; their stability can be inferred from the arrows representing the dynamics of the system. When $u/k = 10$ (weak gene expression,

Fig. 2a) there are two equilibria, at (0, 0) and (1, 1), of which the first is unstable and the second stable; we predict equal tRNA abundances and little bias in codon usage. When $u/k = 0.1$ (strong gene expression, Fig. 2b) there are three equilibria, at (0, 0), (0.113, 0.013) and (1, 1), of which the first and third are unstable and the second stable; we expect to see unequal tRNA abundances and strong bias in codon usage.

The solid curve lies above the dashed curve near the origin for all values of u/k because its slope at the origin is u/k whereas the dashed curve is horizontal. Whether the system behaves as shown in Fig. 2a or b depends on whether the solid curve approaches (1, 1) from above or below the dashed curve. Evaluating the slopes of the curves at this point shows that this depends on whether u/k is greater or less than 0.25. When u/k is small the stable equilibrium is approximately $(u/k, (u/k)^2)$.

With variable gene expression, we must plot $\bar{q}/\bar{p}$ against C_2/C_1 . Equations (1) and (3) allow q_i to be calculated in each category of gene expression determined by k_j , whence $\bar{q}/\bar{p}$ can be calculated to give the solid curve; the dashed curve is unchanged. The argument used in the previous paragraph shows that the system behaves as in Fig. 2a or b according as u/k is greater or less than 0.25. Note that $\bar{k}$ is a weighted average, $\bar{k} = \sum_j w_j k_j$, so that strongly expressed genes are highly influential in determining the behaviour of the system.

This result provides a possible explanation for the existence of highly biased codon usage associated with unequal tRNA abundances in unicellular organisms in which rapid clonal division is an important component of fitness so that there is strong selection pressure to minimize translation time. To obtain biased codon usage under this model the weighted mean selec-

tion pressure per nucleotide, $\bar{k}$, in response to tRNA abundance must exceed both the mutation rate per nucleotide, u , and the reciprocal of the population size¹², otherwise codon usage is largely determined by mutation pressure. Data on the relationship between codon usage and tRNA abundance¹ (Fig. 1) show that $u/\bar{k}$ is small in both *E. coli* and yeast, so that coupled with their large population size they meet the requirements for biased codon usage. Multicellular organisms are likely to have both lower selection pressure at the level of translation and smaller population size, consistent with the conclusion that their codon usage is heavily influenced by mutation pressure¹.

Several simplifying assumptions have been made in developing the model, but it seems unlikely that the conclusions would be qualitatively changed by relaxing them, except for the assumption in obtaining equation (5) that selection pressure is inversely proportional to tRNA abundance.

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- Ikemura, T. *Mol. Biol. Evol.* **2**, 13-34 (1985).
- Post, L. E., Strycharz, G. D., Nomura, M., Lewis, H. & Dennis, P. P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1697-1701 (1979).
- Bennetzen, J. L. & Hall, B. D. *J. biol. Chem.* **257**, 3026-3031 (1982).
- Kimura, M. *The Neutral Theory of Molecular Evolution* (Cambridge University Press, Cambridge, 1983).
- Sharp, P. M. & Li, W.-H. *J. molec. Evol.* (in the press).
- Varenne, S., Buc, J., Llobes, R. & Lazdunski, C. *J. molec. Biol.* **180**, 549-576 (1984).
- Pedersen, S. *EMBO J.* **3**, 2895-2898 (1984).
- Robinson, M. *et al. Nucleic Acids Res.* **12**, 6663-6671 (1984).
- Bonekamp, F., Andersen, H. D., Christensen, T. & Jensen, K. F. *Nucleic Acids Res.* **13**, 4113-4123.
- Gouy, M. & Grantham, R. *FEBS Lett.* **115**, 151-155 (1980).
- Lipman, D. J. & Wilbur, W. J. *J. molec. Biol.* **163**, 363-376 (1983).
- Li, W.-H. *J. molec. Evol.* (in the press).

Structure of D-ribulose-1,5-bisphosphate carboxylase/oxygenase from *Alcaligenes eutrophus* H16

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RuBPCase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC4.1.1.39) is the key enzyme of the reductive pentose phosphate cycle. Because of its biological significance, many structural studies on a number of plant and bacterial RuBPCases¹ have been undertaken, including the enzyme isolated from the autotrophic hydrogen-oxidizing bacterium *Alcaligenes eutrophus* H16 (refs 2-6). Although both the higher plant enzyme and the *A. eutrophus* enzyme consist of eight large and eight small subunits (L_8S_8), no model describing the quaternary structure is generally accepted. Here we present a model for the *A. eutrophus* RuBPCase derived from X-ray crystallography of three-dimensional (3D) crystals, and electron microscopy and image analysis of two-dimensional (2D) crystals of the enzyme. The X-ray electron density of RuBPCase in the presence of HCO_3^- , Mg^{2+} , and the transition state analogue 2-carboxyarabinitol-1,5-bisphosphate (CABP) shows an L_8S_8 molecule in which the L_4S_4 half molecules have local 4-fold symmetry (C4). The local 4-fold axes of the two L_4S_4 halves do not coincide but are shifted by 36 Å and are related by a crystallographic 2-fold axis perpendicular to and between the local 4-fold axes. Electron microscope data of the enzyme without CABP, which can be perfectly modelled using the X-ray densities, do not show this shift and the low-resolution point group of the molecules in the 2D crystals is D4. Both structures are presented.

A variety of approaches to analysing the major structural aspects of the tobacco and the *A. eutrophus* RuBPCase have led to the consensus that the molecule has an L_8S_8 stoichiometry, a diameter of around 125 Å and a height of around 100 Å. These studies include electron microscopy of negatively stained^{2,7} and antibody-labelled⁴ single molecules, and thin⁸ and sectioned^{5,7} 3D crystals, as well as preliminary X-ray crystallographic work^{5,7-9}. Some crystal forms have 1/4 molecule per asymmetric unit⁷ indicating a minimal point group symmetry D2; others have point group symmetry C4⁸ or even D4⁹. Problems associated with X-ray studies on *A. eutrophus* RuBPCase include, for example, twinned crystals of apparent space group $P4_212_1^{5,6}$. The current models for L_8S_8 RuBPCase from tobacco and *A. eutrophus* show 4-fold rotational symmetry^{2,8,9}, but differ in spatial arrangement of the subunits.

The enzyme exists in two different activation states: high activity is associated with the presence, and inactivity with the absence, of HCO_3^- and Mg^{2+} ions¹⁰. The change in activation state is paralleled by a change in enzyme configuration¹¹ and in sedimentation behaviour of the enzyme (active: 14.3S; inactive: 17.5S)¹⁰.

For X-ray diffraction studies, large single crystals of *A. eutrophus* RuBPCase were grown in the presence of HCO_3^- , Mg^{2+} and CABP⁶. The space group C22₁ and unit cell constants $a = 158.8$ Å, $b = 159.6$ Å, $c = 201.2$ Å imply that the asymmetric unit contains half an L_8S_8 molecule; the other half is related by a crystallographic 2-fold rotation axis. X-ray diffraction data of

Table 1 Quality of SIR phases

	16.67	12.50	10.00	8.33	7.14	6.25	5.65	5.00	Total
Resolution (Å)	16.67	12.50	10.00	8.33	7.14	6.25	5.65	5.00	
Figure of merit	0.67	0.58	0.70	0.63	0.59	0.55	0.47	0.44	0.53
Phasing power*	1.71	1.73	1.82	1.72	2.03	2.16	1.95	1.73	1.88
Anomalous dispersion†	2.13	1.83	2.01	1.78	1.46	1.33	1.29	1.23	1.32

* Phasing power = r.m.s. (f_H/E), where f_H is the calculated heavy atom structure factor amplitude and E is the closure error.

† Anomalous dispersion is the anomalous dispersion difference divided by the estimate of the error.

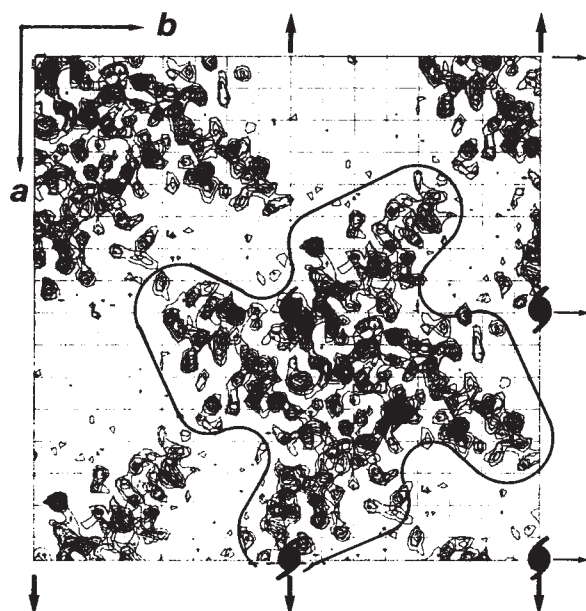


Fig. 1 Electron density sections parallel to the a, b plane. Crystallographic symmetry axes are indicated. Only one half of the unit cell, in the c direction, is shown. Data were collected by the rotation method. The native and HgCl_2 -derivatized 3.2-Å data sets gave R_{merge} values of 0.129 for 36,933 reflections (native) and 0.158 for 36,129 reflections (derivative), where $R_{\text{merge}} = \sum(I(i) - \langle I \rangle) / \langle I \rangle$, where $I(i)$ is the intensity of an individual measurement and $\langle I \rangle$ is the average value. The heavy atom positions were determined by difference Patterson methods. Once the heavy atom coordinates were obtained and refined (least squares and phase refinement), SIR phases were calculated (Table 1) and used to compute a 5-Å-resolution electron density map. Then, solvent flattening, map averaging, and phase combination procedures were used which converged at an R factor of 16% for agreement between structure amplitudes calculated from the modified electron density map and observed data, an average figure of merit of 0.82, and an accumulated phase shift of 58.1°.

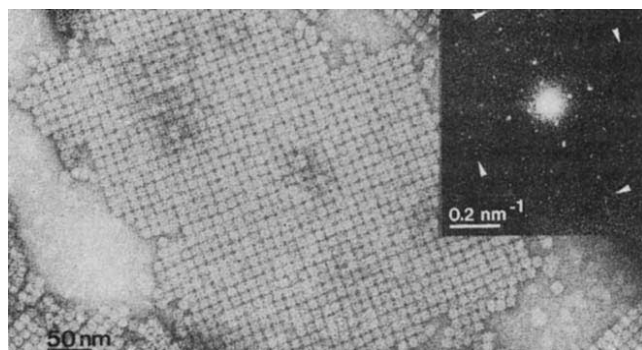


Fig. 2 Electron micrograph of a two-dimensional crystal negatively stained with a 4% (w/v) aqueous solution of uranyl acetate (pH 4.5) and imaged at 80 kV and 50,000× magnification. The insert shows its optical diffraction pattern. The diffraction maxima extend up to the fifth order (~ 23 Å) in one direction, and up to the fourth order (~ 29 Å) in the other direction (white arrows). This difference is due to slight lattice distortions that can be corrected for by correlation averaging.

native and HgCl_2 -derivatized crystals prepared by soaking were collected on films to 3.2-Å resolution. The Patterson rotation function, calculated with vectors in the 25–7 Å range, demonstrates the presence of a local 4-fold axis parallel to the crystallographic c axis, and a local 2-fold axis in the a, b plane. The structure was solved to 5-Å resolution by the single isomorphous replacement (SIR) method, considering anomalous scattering contributions (Table 1). The electron density map (Fig. 1) was improved using solvent flattening^{12,13} followed by 4-fold rotation

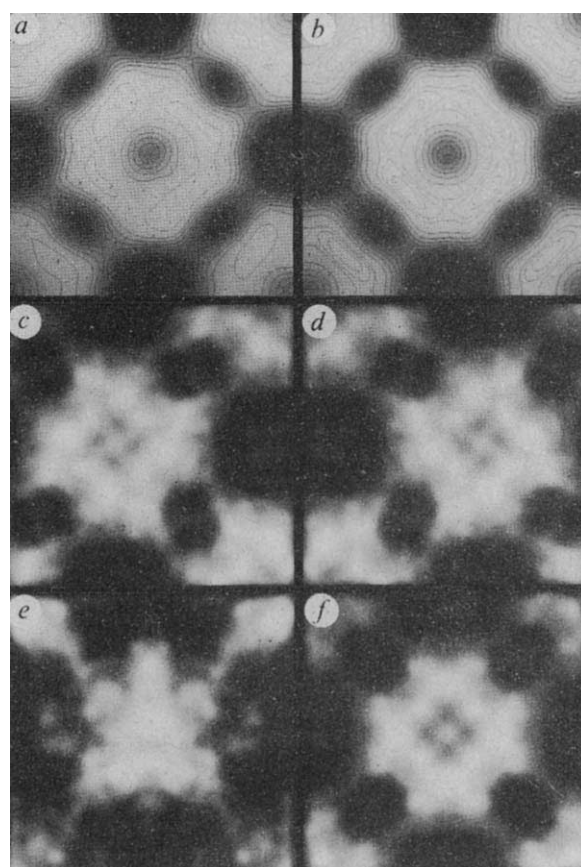


Fig. 3 Computer image analysis of electron micrographs (a, b) and comparison with X-ray electron density ($c-f$). a , Correlation averaging result with 17-Å resolution; b , the image in a with 4 mm symmetry imposed. c, d , Projections through the top and lower half molecules (each L_4S_4). e , Total projection (top and bottom) through the C_2 molecule; f , total projection (top and bottom) through the molecule after removal of the 36-Å shift between half molecules. Full 4 mm symmetry is exhibited.

averaging and phase combination¹³. The crystallographic 2-fold rotation axis along a relates the top and the bottom halves of the L_8S_8 molecule to each other and introduces a 36-Å shift between the two halves as the local 4-fold symmetry axes are 18 Å away from the twofold axis parallel to a (Fig. 4a); the point group thus reduces to C_2 . The L_4S_4 half of the molecule displays four protrusions (Fig. 1) and a central depression is observed in upper layers of the electron density map (Fig. 4).

For electron microscopy, *A. eutrophus* H16 (DSM 428) RuBPCase was purified² using autotrophically grown¹⁴, RuBPCase derepressed¹⁵ cells. Purity of the fully active enzyme was greater than 98%, as determined by analytical SDS polyacrylamide gel electrophoresis¹⁶. The carboxylase activity was measured by the incorporation of $^{14}\text{CO}_2$ into acid-stable product at 30 °C². Using a modification of a previously described method for the spinach RuBPCase¹⁷, the enzyme was crystallized into a monolayer directly on the carbon support film used for electron microscopy. For crystallization the buffer was equivalent to that applied throughout the purification. However, it was supplemented with ammonium sulphate up to 7.77% saturation; it contained HCO_3^- and Mg^{2+} ions necessary to maintain RuBPCase activity.

The plane group of the 2D crystals in projection (Fig. 2) appears tetragonal ($p4mm$) with lattice dimension $a = 117 \pm 5$ Å. Diffraction maxima up to the fifth order (~ 23 Å) can be detected. The micrograph shown in Fig. 2 was analysed by correlation averaging¹⁸ with the IMAGIC image processing system¹⁹. In this technique, the image of the 2D crystal is correlated with respect to a small reference crystal patch. The cross-correlation

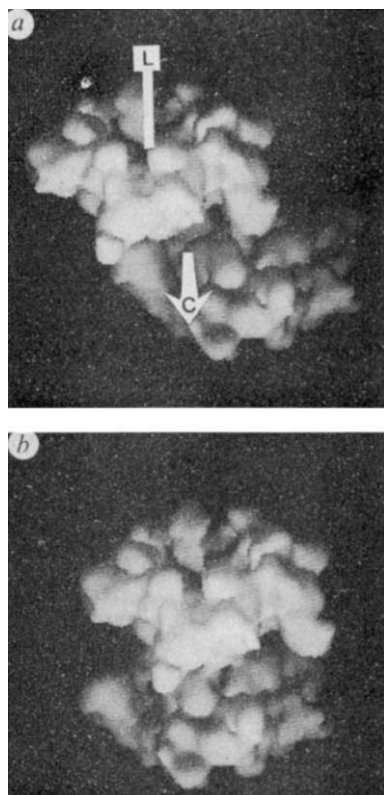


Fig. 4 Surface representation of the enzyme configuration derived from the 5-Å electron-density map of *Alcaligenes eutrophus* RuBPCase, where local 4-fold (L) and crystallographic 2-fold (C) symmetry elements are marked. *b*, Surface representation of the D4 model calculated from the X-ray electron density map after undoing the 36-Å shift between half molecules. In both *a* and *b*, the X-ray electron density map was low-pass filtered to 20-Å resolution to emphasize the quaternary structure. A surface representation algorithm was used^{23,24}.

map resulting from this operation contains peaks at the best match positions which also correspond to the positions of the individual unit cells. Using the coordinates of these peaks, the individual unit cells are extracted from the crystal and summed to give an image with an enhanced signal-to-noise ratio. Figure 3*a* shows the average of 900 aligned unit cells with a reproducible resolution of 17 Å as determined by Fourier ring correlation^{18,20}. Because correlation averaging removes crystal distortions by translational alignment, the resolution of the final sum is better than could be achieved by simple Fourier peak filtering (17 Å compared with 23 Å)^{18,19}.

The average image (Fig. 3*a*) exhibits 4-fold symmetry even before 4mm symmetrization (Fig. 3*b*). The molecule has a central stain pit and is connected to its neighbours by eight 'arms'. The 4-fold symmetry indicates that the distribution of negative stain is almost uniform throughout the molecule, and is not restricted to only one half (L_4S_4). In contrast, molecules within 2D crystals rotationally shadowed with platinum-carbon show a strong handedness (data not shown). The electron micrographs analysed here of negatively stained specimens represent projections throughout the entire structure, as has been previously demonstrated for other molecules²¹.

The lattice repeat of 117 ± 5 Å of the 2D crystal agrees well with the crystallographic data, where 112.4 Å is half of the crystal diagonal with $a = 158.8$ Å and $b = 159.6$ Å, suggesting that the lateral molecular packing in the 2D and 3D crystals is similar. X-ray density maps of the top and bottom halves of the molecule were blurred to 12 Å resolution and projected along the *c* axis (Fig. 3*c, d*) for comparison with electron microscope projections. Summing both halves gives a total projection

through the structure (Fig. 3*e*) which only exhibits mirror symmetry, because the projection is perpendicular to the 2-fold axis of the C2 structure. When each half is shifted by 18 Å to make the local 4-fold axes coincide, the projection through the whole molecule becomes 4-fold symmetric (4 mm) (Fig. 3*f*) and corresponds closely with the electron microscope projections (Fig. 3*a, b*). We conclude that the point symmetry of RuBPCase complexed with the transition state analogue CABP in the C222₁ 3D crystal and of RuBPCase without CABP in the p4 2D crystal are essentially different (C2 and D4 respectively) (Fig. 4). Despite the introduction of the lateral shift between the half molecules (Fig. 4*a*), the nature of the lateral intermolecular contacts in both crystals remains almost identical.

Further crystallographic studies seek to derive a detailed molecular structure to higher (3.2-Å) resolution. However, even at the present resolution level (X-ray, 5 Å; EM, 17 Å), good agreement between results obtained independently using different approaches emphasizes the reliability of our data and leads to the conclusion that we have studied the molecule in two different structural states. In the C222₁ crystals, the 36-Å shift between the half molecules may have been induced by the presence of CABP. The alternative explanation, that the 3D crystal was composed of L_4S_4 half molecules with a non-crystallographic contact forming the octamers could be excluded: using low-speed sedimentation equilibrium centrifugation⁴ the enzyme was shown not to decompose from its hexadecameric to an octameric state even at 25% ammonium sulphate saturation. Moreover, the idea that a lateral shift between half molecules may occur is supported by (1) independent X-ray studies with the tobacco enzyme⁹ which indicate that the molecule can also assume D4 point group symmetry in 3D crystals obtained without HCO_3^- , Mg^{2+} and CABP; (2) biochemical studies on the spinach RuBPCase revealing different chromatographic and electrophoretic properties for the active and the CABP-treated enzyme²²; and (3) preliminary electron microscope results performed on freeze-dried preparations of the enzyme complexed with CABP (data not shown). The relation between the two forms of RuBPCase and the active and inactive states of the enzyme remains to be investigated.

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- Miziorko, H. M. & Lorimer, G. H. *Rev. Biochem.* **52**, 507-535 (1983).
- Bowien, B., Mayer, F., Codd, G. A. & Schlegel, H. G. *Arch. Microbiol.* **110**, 157-166 (1976).
- Purohit, K. & McFadden, B. A. *Biochem. biophys. Res. Commun.* **71**, 1220-1227 (1976).
- Bowien, B. & Mayer, F. *Eur. J. Biochem.* **88**, 97-107 (1978).
- Bowien, B. *et al. Eur. J. Biochem.* **106**, 405-410 (1980).
- Pal, G. P., Jakob, R., Hahn, U., Bowien, B. & Saenger, W. *J. biol. Chem.* **260**, 10768-10770 (1985).
- Baker, T. S., Eisenberg, D., Eiserling, F. A. & Weissman, L. *J. molec. Biol.* **91**, 391-399 (1975).
- Baker, T. S., Eisenberg, D. & Eiserling, F. A. *Science* **196**, 293-295 (1977).
- Baker, T. S., Suh, S. W. & Eisenberg, D. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1037-1041 (1977).
- Bowien, B. & Gottschalk, E.-M. *J. biol. Chem.* **157**, 11845-11847 (1982).
- Meisenberger, O., Pilz, I., Bowien, B., Pal, G. P. & Saenger, W. *J. biol. Chem.* **259**, 4463-4465 (1984).
- Wang, B. C. in *Lecture Notes for the School on Direct Methods and Macromolecular Crystallography*, 1-7 (Medical Foundation of Buffalo, New York, 1983).
- Rossmann, M. G. & Blow, D. M. *Acta crystallogr.* **15**, 24-31 (1962).
- Schlegel, H. G., Kaltwasser, H. & Gottschalk, G. *Arch. Microbiol.* **38**, 209-222 (1961).
- Friedrich, C. G. *J. Bact.* **149**, 203-210 (1982).
- Weber, K., Pringle, J. R. & Osborne, M. *Meth. Enzym.* **26**, 3-27 (1972).
- Barcena, J. A. & Shaw, P. J. *Planta* **163**, 141-144 (1985).
- Saxton, W. O. & Baumeister, W. *J. Micro.* **127**, 127-138 (1982).
- Van Heel, M. & Keegstra, W. *Ultramicroscopy* **7**, 113-130 (1981).
- Van Heel, M. & Stöfner-Meilicke, M. *EMBO J.* **4**, 2389-2395 (1985).
- Johannsen, W., Schütte, H., Mayer, F. & Mayer, H. *J. molec. Biol.* **134**, 707-726 (1979).
- Johal, S., Partridge, B. E. & Cholet, R. *J. biol. Chem.* **260**, 9894-9904 (1985).
- Van Heel, M. *Ultramicroscopy* **11**, 307-314 (1983).
- Saxton, W. O. *Ultramicroscopy* **16**, 387-394 (1985).

The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor

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Alzheimer's disease¹ is characterized by a widespread functional disturbance of the human brain. Fibrillar amyloid proteins are deposited inside neurons as neurofibrillary tangles² and extracellularly as amyloid plaque cores² and in blood vessels². The major protein subunit (A4) of the amyloid fibril of tangles, plaques and blood vessel deposits is an insoluble, highly aggregating small polypeptide of relative molecular mass 4,500³⁻⁶. The same polypeptide is also deposited in the brains of aged individuals with trisomy 21 (Down's syndrome)^{3,5,6}. We have argued previously that the A4 protein is of neuronal origin and is the cleavage product of a larger precursor protein^{4,6}. To identify this precursor, we have now isolated and sequenced an apparently full-length complementary DNA clone coding for the A4 polypeptide. The predicted precursor consists of 695 residues and contains features characteristic of glycosylated cell-surface receptors. This sequence, together with the localization of its gene on chromosome 21, suggests that the cerebral amyloid deposited in Alzheimer's disease and aged Down's syndrome is caused by aberrant catabolism of a cell-surface receptor.

We reasoned that the precursor of the A4 polypeptide may be produced in large amounts during the whole of life by cells of the human central nervous system. We therefore used brain tissue of a five-month-old fetus without trisomy 21 to obtain the messenger RNA needed to construct a cDNA library⁷. We used a probe designed from the N-terminal part of the A4 polypeptide to screen the library (details in Fig. 1 legend), and found several positive clones. The first one sequenced (clone 9-110) encoded the entire A4 subunit protein.

If we assume that translation starts at the first AUG, the protein would have 695 residues. It begins with a signal sequence (Figs 1 and 2) for transport through the endoplasmic reticulum membrane⁸. The signal sequence is followed by a region rich in

cysteine. The last of 12 cysteines occurs at residue 187. Thus, the exact folding of these N-terminal regions may be stabilized by disulphide bridges. The next hundred residues are of peculiar composition. They include a stretch of seven uninterrupted threonine residues and are extremely rich in glutamic (28 residues) and aspartic (17 residues) acids, but contain only one lysine and two arginines. This domain would be able to bind large numbers of positive ions or polycations and may function in the electrical activity of nerve cells. The region from residue 290 to residue 597, the N-terminus of the amyloid A4 protein, contains two potential N-glycosylation sites at positions 467-469 and 496-498.

Like the anionic domain, the sequence of the amyloid A4 protein is also unique. We determined the complete amino-acid sequence of the A4 polypeptide isolated from both amyloid plaque cores and neurofibrillary tangles (for details see Fig. 2 legend). At most the amyloid subunit can be either 42 or 43 residues long and shows N-terminal heterogeneity as expected from previous work^{3,4,6}. The 43-residue protein terminates at, and the 42-residue protein terminates just before, the first of the two threonine residues found in the potential transmembrane region (residues 625-648). The C-terminal cleavage of mature precursor during amyloidogenesis would thus have to occur within the membrane. Such a cleavage seems unlikely and the details of this process deserve further attention. The C-terminal end of the precursor is short (57 residues) compared to the cytoplasmic domain of known receptor proteins⁹. Following the putative membrane domain, there are three lysine residues (649-651) which probably interact with the phospholipid head groups in the membrane and are a characteristic feature for the junction between membrane and cytoplasmic domains of cell-surface receptors⁹. There is also a single sequence capable of asparagine-linked glycosylation (Asn-Pro-Thr). However, it is likely that this site is not glycosylated because of the presence of proline (Figs 1 and 2). The sequence and composition of the A4 precursor are not homologous to known proteins (see Fig. 1 legend) including the sequenced components of normal neurofilaments^{10,11} or the PrP protein and gene isolated from the transmissible spongiform encephalopathies¹².

To determine the size of the mRNA encoding the A4 precursor we performed Northern blot hybridizations¹³. Total RNA was isolated from the same fetal cortex used for constructing the cDNA library. The RNA preparation was probed with the *Eco*RI fragment of the cloned A4 precursor cDNA (Fig. 3a). Two transcripts of 3.4 and 3.2 kilobases (kb) were detected. Both signals are in the size range of the cDNA insert described in Fig. 1. Similar results were obtained with mRNA from normal adult human cortex, and from cortex of sporadic Alzheimer's disease patients (data not shown).

Southern blot analyses¹⁴ of DNA from 29 human × mouse cell hybrids¹⁵ were used to localize human A4 precursor sequen-

Table 1 Chromosome location of human A4 precursor gene

Lane in Fig. 3b	Hybrid clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Hybridization (8.8-kb band) in Fig. 3b
1	Human genome	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	+
2	RAG-SU 3-1-2-1			O	X	X	O	O															X	X		+
3	RAG RU 4-13	O		X		O	X	O		O				O				X		O		X	X	O	X	+
4	RAG 194-7	X		O	X	X			X	X		O								X		X		O		+
5	A9-SU 1-2			X	O		X							X	X				X			X				+
6	MS2 B82-1a-141		X	X	X			X	X			X		X		X	X	X	X	X		X	X	X	X	+
7	RAG ANLY 1	X		X	X	X	X						X		O				X			X				+
8	RAG 194-5-5			O	X	X	X	X		X			X	X	X		X				X	X		O		+
9	RAG GO-4			X	X			X	X					X	X	X			X				X	O		-
10	RAG PI 7-2	X		X		X	X	X	X			O	X		X	O			X				X	O		-
11	RAG 610-4-5-1	O		X	X	X	O						X	O	X	X			O		X		X	X		-
12	RAG mouse																									-

X, whole chromosome; O, chromosome fragment.

Fig. 1 Nucleotide sequence and predicted amino-acid sequence of a cDNA clone encoding the precursor of the amyloid A4 protein of Alzheimer's disease. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first base of the initiation codon AUG. The untranslated sequence directly following the poly(G) tail is indicated by negative numbers. The sequence shows a 695-residue open reading frame. The deduced amino-acid sequence is numbered, the amino-terminal methionine included. The amino-acid sequence of the A4 polypeptide (includes data from refs 3 and 4) is boxed. Zigzag underline, a probable membrane-spanning sequence of the A4 polypeptide. The synthetic oligonucleotide mixture used as probe is indicated as a line above the corresponding cDNA. Polyadenylation signals are underlined. Nucleotide 3207 is followed by a poly(dA) tail linked to the vector DNA. A computer search for homology was unsuccessful using computer programs of the University of Wisconsin Genetics Computer Group (UWGCG). The entire cDNA insert includes 3,353 bp and *EcoRI* sites at bp positions 1,795 and 2,851. The amino-acid composition of the 695-residue A4 precursor is A57, C12, D47, E85, F17, G31, H25, I23, K38, L52, M21, N28, P31, Q33, R33, S30, T45, V62, W8, Y17 resulting a calculated M_r of 78,644.45.

Methods. Total RNA was extracted²⁴ from cortex of the brain of a 5-month-old aborted human fetus. Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose chromatography²⁵. A cDNA library was constructed by the method of Okayama and Berg⁷ with 3 μ g poly(A)⁺ RNA and 1.5 μ g vector-primer DNA. The cDNA was transformed into *Escherichia coli* HB101. Transformants (10^5) were screened²⁶ with a probe consisting of a mixture of 64 20-mers, 5'TT⁻TC⁻TA⁻GT⁻AG⁻TGCAC⁻T⁻CA⁻TA3', deduced from residues 10-16 of the A4 polypeptide. Of the nine positive clones obtained, one (clone 9-110) was sequenced by the dideoxy chain termination method²⁷ on both strands with the help of synthetic primers. The primers were synthesized on a DNA-synthesizer (Applied Biosystems)²⁸. For sequence comparison, UWGCG programs Wordsearch and Gap were applied to the data libraries of EMBL and NBR7.

Fig. 2 Model of A4 precursor as a cell-surface glycoprotein. **a**, Amino-acid sequence of amyloid plaque core-A4 polypeptide. Amyloid from plaque cores was purified from human post-mortem brain homogenates^{3,6}. The amino-terminal sequence shown (A) was obtained for amyloid plaque core-A4 polypeptides. To determine the internal and the C-terminal portion of the A4 polypeptide, the corresponding trypsin digestion (B) and cyanogen bromide cleavage products (C, D) were sequenced. However, we were unable to assign residues 32 and 33 unambiguously. Amyloid plaque core preparations from different Alzheimer brains suggest that the amyloid protein may consist variably of at most 42 or 43 residues (C, D).

b, Proposed domain structure: black box, signal sequence⁸, open box, cysteine-rich region; hatched box, highly negatively charged domain (45% Asp and Glu residues); filled circles, N-glycosylation sites. The A4 amyloid is shown in black between the two arrowheads and reaches into the (boxed) hydrophobic transmembrane segment. The putative cytoplasmic domain includes the sequence Asn-Pro-Thr (open circle).

c, The full-length A4 amyloid protein (42–43 residues) as deposited in the brain of patients with Alzheimer's disease (shaded region), includes 28 residues of the extracellular domain and 14–15 residues of the transmembrane domain of the A4 precursor. The group of three Lys residues following the 24 hydrophobic amino acids of the putative transmembrane domain are typical for the junction between membrane and cytoplasmic domains of cell surface receptors⁹.

Methods. The amyloid plaque cores were extracted with formic acid as described^{3,4} and the formic acid-soluble protein was applied to the gas-phase protein sequencer (Model 470 A, Applied Biosystems) described by Hewick *et al.*²⁹. The amino-acid derivatives released were determined by reverse-phase HPLC in on-line configuration using 40% of the sample (Model 120A, Applied Biosystems). Four different amyloid plaque core preparations were sequenced; typically, 400 pmol of total N-terminal derivatives was obtained from 900 pmol of protein (estimated by quantitative amino-acid analysis). For the tryptic digest in **b**, 10 pmol of sequence was obtained from the estimated 5 nmol of acetylated protein; 20 pmol (**c**) and 50 pmol (**d**) of sequence was obtained for the cyanogen bromide cleavages from an estimated 2 nmol of acetylated protein (details to be published elsewhere).

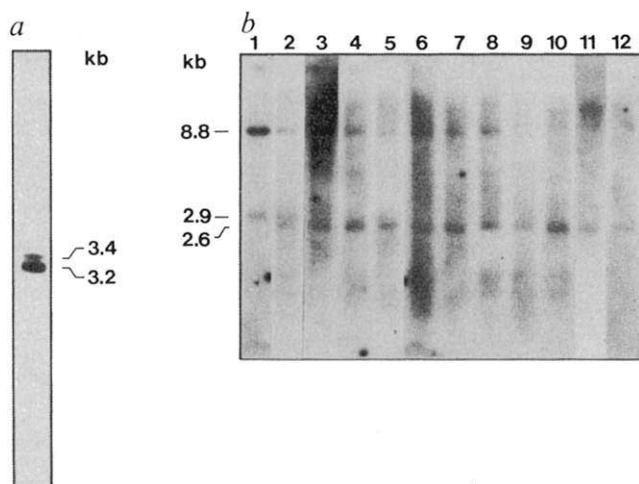
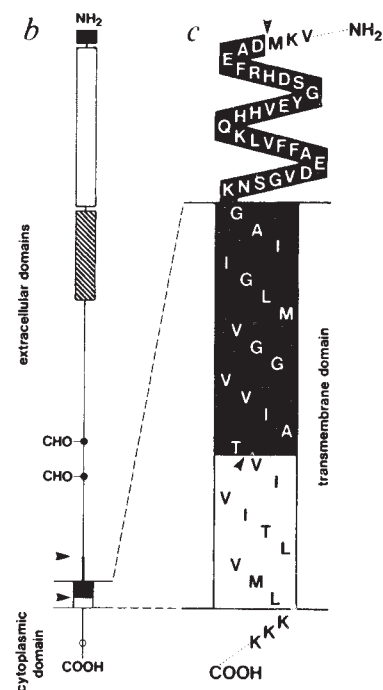
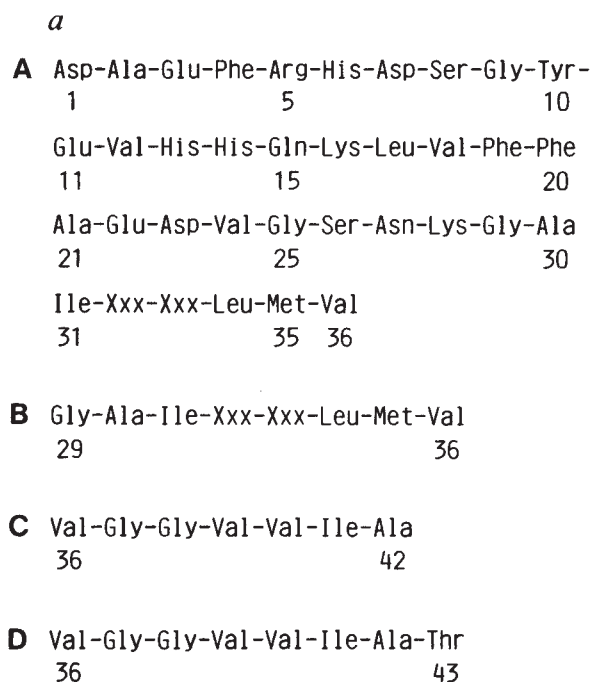


Fig. 3 Hybridization analyses. **a**, Identification of A4 precursor mRNA by Northern blot analysis¹³. RNA was isolated from human embryonic cortex as in Fig. 1. RNA (25 µg) was heated for 15 min at 65 °C in the presence of 50% formamide, subjected to electrophoresis on a 1.6% agarose gel containing 6 M urea/25 mM sodium citrate, pH 3.5³⁰ and transferred to Zetabind membrane (AMF Cuno). Blots were hybridized at 65 °C with nick-translated³¹ 1,056-bp *Eco*RI fragment of clone 9-110 (bp positions 1,795–2,851, Fig. 1) in 6 × SSC, 50 mM sodium phosphate pH 6.5, 5 × Denhardt's, 0.5% SDS, 1 mM EDTA, 100 µg ml⁻¹ denatured calf thymus DNA. The filters were washed to a final stringency of 0.2 × SSC, 0.1% SDS at 65 °C. Autoradiography was for 24 h at –70 °C using intensifying screens. An RNA ladder (BRL) was used as size marker. **b**, Chromosome mapping of A4 precursor locus. The 1,056-bp cDNA *Eco*RI fragment of clone 9-110 (Fig. 1) was used for Southern blot analysis¹⁴ of *Eco*RI-digested human × mouse RAG or mouse A9 hybrid cell line DNAs. DNA from the somatic cell hybrids was purified from isolated nuclei as described in detail elsewhere¹⁵. The *Eco*RI-digested DNA (~10 µg) from each of 29 hybrid cell samples was electrophoresed through a 0.8% agarose gel and blotted onto nylon (Pall). Results from 10 of the 29 hybrids analysed are presented here and in Table 1. Hybridization and washing were as in **a**. Lane 1, human genomic DNA; lanes 2–8, hybrid cell lines containing human chromosome 21; lanes 9–11, hybrid cell lines missing human chromosome 21; lane 12, mouse RAG genomic DNA. The chromosome distribution is given in Table 1.

ces to chromosome 21. The ³²P-labelled *Eco*RI fragment of the human A4 precursor cDNA was hybridized to Southern filters of *Eco*RI-digested DNA from control tissue and hybrid cell lines. Two human-specific fragments of 8.8 and 2.9 kb and a mouse-specific fragment of 2.6 kb were observed (Fig. 3b). The 8.8 kb fragment was used to score for the presence of the human A4 precursor gene in the DNA from the hybrid cell lines. Chromosome 21 was the only human chromosome that showed perfect concordance with human A4 precursor sequences

(Fig. 3b, lanes 2–8, and Table 1). Every other human chromosome could be ruled out by at least two discordant hybrids.

It may be significant that chromosome 21 is the chromosome that is duplicated in Down's syndrome. We have evidence that the A4 gene is close to the part of the chromosome that is duplicated (unpublished results). There could be a more complex interaction between other chromosome 21 gene products and the biosynthesis and processing of the A4 precursor in Down's syndrome, rather than a simple gene-dosage effect.

The transmissible spongiform encephalopathies, scrapie, CJD and kuru¹⁶ are progressive degenerative disorders of the nervous system sharing many clinical and pathological features with Alzheimer's disease. In these unusual infectious diseases, an abnormally post-translationally modified protein (PrP)¹² accumulates in the form of amyloid structures in the brain¹⁷⁻²⁰. However, the corresponding gene for the PrP protein precursor has been localized to chromosome 20²¹. Nevertheless, we and Oesch *et al.*¹² show that the two proteins share many similarities, including features characteristic of glycosylated membrane receptors. It is possible that the putative membrane-spanning domains of both proteins share an amyloid-forming or amyloid-inducing potential.

The availability of a complete A4 precursor cDNA now permits a systematic study of the normal and abnormal biology of this amyloidogenic protein in Down's syndrome, familial and sporadic Alzheimer's disease and related disorders. During the preparation of this manuscript we have learnt that two other groups have isolated cDNA clones coding for A4^{22,23}.

We thank Robert G. Rohwer and Caroline Hilbich for comments, Klaus Olek for a gift of Okayama-Berg primer, Thomas Herget for RNA, and Brigitte Kisters and Pauline Klein for help with the computers. This work was supported by grants from the Deutsche Forschungsgemeinschaft through SFB 74 A1, A2 and GR 373, and the National Health and Medical Research Council of Australia. The sequence data in this publication have been submitted to the EMBL/GenBank Libraries under the accession number Y00264.

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1. Alzheimer, A. *Allg. Z. Psychiat.* **64**, 146-148 (1907).
2. Katzman, R. (ed.) *Biological Aspects of Alzheimer's Disease (Banbury Report 15)* (Cold Spring Harbor Laboratory, New York, 1983).
3. Masters, C. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 4245-4249 (1985).
4. Masters, C. L. *et al. EMBO J.* **4**, 2757-2763 (1985).
5. Glenner, G. G. & Wong, C. W. *Biochem. biophys. Res. Commun.* **120**, 1131-1135 (1984).
6. Beyreuther, K. *et al. in Discussions in Neurosciences* Vol. 3 (eds Bignami, A., Bolis, L. & Gajdusek, D. C. 68-79 Fondation pour l'Etude du Système Nerveux, Geneva (1986).
7. Okayama, H. & Berg, P. *Molec. cell. Biol.* **3**, 280-289 (1983).
8. Blobel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 852-862 (1975).
9. Yarden, Y. *et al. Nature* **323**, 226-232 (1986).
10. Geisler, N., Plessmann, U. & Weber, K. *FEBS Lett.* **182**, 475-478 (1985).
11. Lewis, S. A. & Cowman, N. J. *Molec. cell. Biol.* **6**, 1529-1534 (1986).
12. Oesch, B. *et al. Cell* **40**, 735-746 (1985).
13. Thomas, P. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
14. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
15. Löttscher, E. *et al. Nature* **320**, 456-458 (1986).
16. Gajdusek, D. C. *Science* **197**, 943-960 (1977).
17. Multhaup, G. *et al. EMBO J.* **4**, 1495-1501 (1985).
18. Merz, P. A., Sommerville, R. A., Wisniewski, H. M. & Iqbal, K. *Acta neuropath.* **54**, 63-74 (1981).
19. Düringer, H. *et al. Nature* **306**, 476-478 (1983).
20. Prusiner, S. B. *et al. Cell* **35**, 349-358 (1983).
21. Liao, Y.-C. J., Lebo, R. V., Clawson, G. A. & Smuckler, E. A. *Science* **233**, 364-367 (1986).
22. Goldgaber, D., Lerman, M. I., McBride, W. O., Sallow, V. & Gajdusek, D. C. *Science* (in the press).
23. Robakis, N. K. *et al. Lancet* (manuscript in preparation).
24. Bernstein, S. L., Gioco, A. E. & Kaplan, B. B. *J. Neurogenet.* **1**, 71-86 (1983).
25. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408-1412 (1972).
26. Woods, D. E., Markham, A. F., Ricker, A. T., Goldberger, G. & Colten, H. R. *Proc. natn. Acad. Sci. U.S.A.* **79**, 5661-5665 (1982).
27. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
28. Matteucci, M. D. & Caruthers, M. H. *J. Am. Chem. Soc.* **103**, 3185-3190 (1981).
29. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. *J. biol. Chem.* **256**, 7990-7997 (1981).
30. Lehrach, H., Diamond, D., Wozney, J. M. & Boedtker, H. *Biochemistry* **16**, 4743-4751 (1977).
31. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).

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Myths and mad March hares

HOLLEY and Greenwood¹ claim that boxing in hares is the female's attempt to prevent mating, and that 'madness' is not a feature of March. The behaviour they describe as boxing I call rebuff². An unreceptive female lunges at a male that comes too close, usually from the semi-crouched threat position. Boxing between males establishes dominance³. In Germany the characteristic erect posture of two males feinting with the fore paws, 'Distanzgefecht' or real boxing in contrast to 'Brustkampf' the usual female rebuff, has been photographed in marked individuals^{4,5}. Dr E. Schneider (in litt., 2/9/84) recorded boxing at least 40 times, all male-male in the 16 bouts with sexes identified. In 17 rebuffs, 14 involved females. That no male-male boxing was recorded by Holley seems curious; perhaps his population was so isolated the males all knew their ranking.

Testosterone levels in English hares rise dramatically from February to April, so interaction between males is likely to peak in March, as do daylight sightings of adult hares in Norfolk⁶. In New Zealand where turf remains short throughout the year, hare sightings peak in September (the equivalent of March). Hence I do not agree that male 'mad March hares' are a myth, or that female hares do the boxing.

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1. Holley, A. J. F. & Greenwood, P. J. *Nature* **309**, 515, 545-550 (1984).
2. Flux, J. E. C. in *Proceedings of the World Lagomorph Conference* (eds Myers, K. & MacInnes, C. D.) 377-394 (University of Guelph, Ontario 1981).
3. Lindlof, B. *Viltrevy* **10**, 145-158 (1978).
4. Schneider, E. in *Ecology and Management of European hare populations* (eds Pielowski, Z. & Pucek, Z.) 41-53 (Polish Hunting Association, Warsaw, 1976).
5. Schneider, E. *Der Feldhase: Biologie-Verhalten Hege und Jagd*. (BLV, München, 1978).
6. Lincoln, G. A. *J. Zool.* **174**, 1-14 (1974).

Sperm sharing in *Biomphalaria* snails

SINCE 1968, as an inference from observations published later¹, I hypothesized that a simultaneous hermaphrodite snail with a planorbid-type reproductive system could transfer sperm from a donor to a recipient snail. Such hypothesis, tested in numerous crossing experiments with *Biomphalaria glabrata*, proved untenable. The report by Monteiro, Almeida and Dias² led me to repeat my former (unpublished, of course) experiments, this time strictly following the procedure described by those authors. I had previously performed unsuccessfully similar experiments at the same laboratory of the University of Brasilia, and with the same strain of *B.*

glabrata successfully handled by these authors.

Concerning *B. tenagophila*, in which transference of sperm from donor to recipient through an intermediate snail is asserted to have occurred in 20 out of 70 experiments (about 28%), I carried out tests with 47 trios, using the same strains that were used by the mentioned authors: NB-JO-JO (14), NB-JO-BH (9), NB-BH-JO (10), JP-JO-JO (7), JP-BH-JO (7). These trios correspond to the most successful combinations mentioned by Monteiro *et al.*, since transference of sperm is said to have occurred in 18 experiments (about 38%). In my trials, however, not even a single candidate recipient produced any embryo resulting from fertilization by donor's sperm during a period of 100 days from the 'intermediate-recipient' pairing. The only explanation that occurs to me for the alleged transference of sperm is that there was confusion of albino 'intermediate' and albino 'recipient' during the mating period, when similar snails were paired and separated every day.

Another disputable statement made by Monteiro *et al.* is that 'crossbreeding between *B. tenagophila* and *B. occidentalis* is common in the laboratory'. In my description of the latter as a new species³, I emphasized that it is indistinguishable from *B. tenagophila* by the shell characteristics, and showed that the two species differ morphologically in some features of the genital system and are separated by absolute reproductive isolation. The latter characteristic was now confirmed, when I failed to interbreed *B. occidentalis* from Sena Madureira (SM) and *B. tenagophila* to form the trios SM-BH-BH, SM-JO-BH and SM-JO-JO, for a complete lack of hybridization between SM-BH (10 couples) and SM-JO (10 couples). My explanation of the successful results mentioned by Monteiro and colleagues is that in their experiments *B. tenagophila* was mistaken for *B. occidentalis*.

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1. Paraense, W. L. *Rev. brasil. Biol.* **36**, 535-539 (1976).
2. Monteiro, W., Almeida, J. M. G. Jr. & Dias, B. S. *Nature* **308**, 727 (1984).
3. Paraense, W. L. *Mems. Inst. Oswaldo Cruz* **76**, 199-211 (1981).

MONTEIRO *et al.*¹ describe sperm sharing in simultaneously hermaphroditic *Biomphalaria* snails, a strategy inconsistent with most models of mating systems. We suggest that this represents 'cheating' in a sperm-trading mating system, such as that found in the opisthobranch *Navanax inermis*²⁻⁴, and predicted for pulmonate gastropods⁴.

Recent theories of mating systems are

based on Bateman's principle⁵, which states that fecundity of females is limited by the energy available for egg production, and that of males by access to females or eggs. Therefore, simultaneous hermaphrodites should mate 'not so much to gain sperm to fertilize eggs as to give sperm away (to gain access to another's eggs)'⁶. That is, they should trade eggs, as has been described for *Hypoplectrus*, a serranid fish⁷. In contrast, sperm sharing represents 'a strategy enabling the snail to receive much sperm from its partners, when it is acting as a functional female, while transferring relatively few spermatozoa of its own'¹. This suggests that *Biomphalaria* are trading sperm rather than eggs.

In a sperm-trading mating system, individuals mate as males not so much to fertilize eggs as to receive sperm to fertilize their own eggs (as in *Navanax*²⁻⁴). Comparison of the mating systems of *Hypoplectrus* and *Navanax* suggests that the risk of gamete loss (rather than the energy invested in gametes) determines the 'preferred' sexual role. Simultaneous hermaphrodites should trade sperm, rather than eggs, wherever sperm are at a greater risk of loss before fertilization³. In *Biomphalaria*, eggs are assured of fertilization because females can self-fertilize if necessary⁸. However, copulating as a male does not guarantee access to eggs since *Biomphalaria* store foreign sperm and digest the excess^{9,10}. To act as a female should therefore be the preferred role in *Biomphalaria*.

Sperm sharing represents a way of reciprocating with a partner without producing any (or as many) sperm of one's own, as these may be digested rather than used in fertilization. It is a way of 'cheating' as a male. Sperm sharing will not harm the female unless the female received its own sperm back from its partner. We expect that mechanisms to prevent this should exist since cross-fertilization is preferred in *Biomphalaria*¹¹.

Monteiro *et al.*¹ suggested that sperm sharing might represent an evolutionarily stable strategy related to a bias towards female function. However, *Hypoplectrus*, which trades eggs, has sex allocation biased toward female function¹² and Bateman's principle¹³ cannot explain why an animal should transfer foreign sperm to a partner.

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1. Monteiro, W., Almeida, J. M. G. Jr & Dias, B. S. *Nature* **308**, 727-729 (1984).

2. Leonard, J. L. & Lukowiak, K. *Am. Nat.* **124**, 282-286 (1984).
3. Leonard, J. L. & Lukowiak, K. *Am. Zool.* **24**, 49A (1984).
4. Leonard, J. L. & Lukowiak, K. *Can. J. Zool.* **63**, 2719-2729 (1985).
5. Bateman, A. J. *Heredity* **2**, 349-368 (1948).
6. Charnov, E. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2480-2484 (1979).
7. Fischer, E. A. *Anim. Behav.* **28**, 620-633 (1980).
8. Brumpt, E. *Ann. Parasit.* **18**, 9-45 (1941).
9. Jong-Brink, M. de. *Z. Zellforsch.* **102**, 507-542 (1969).
10. Kitajima, E. W. and Paraense, W. L. *J. Morph.* **176**, 211-220.
11. Mulvey, M. & Vrijenboek, R. C. *J. Hered.* **72**, 308-312 (1981).
12. Fischer, E. A. *Am. Nat.* **117**, 64-82 (1981).
13. Charnov, E. L. *The Theory of Sex Allocation* (Princeton University Press, 1982).

MONTEIRO ET AL. REPLY—We agree with Leonard and Lukowiak that *Biomphalaria* snails must have reproductive strategies similar to those found in *Navanax*¹ and the finding of sperm trading in these snails will certainly help to explain why sperm sharing should occur². However, egg trading (that is, ovule trading) in *Hypoplectrus* only becomes an evolutionary stable strategy^{3,4} when accompanied by sex allocation biased towards female function. Fischer argues that this is due to decrease in fitness gain in relation to further increments in resources expended in male function⁵, but did not explain why this should happen. Accordingly, we believe sperm trading also needs this type of sex allocation to be an evolutionary stable strategy⁵.

Bias towards female function in both *Hypoplectrus* and *Biomphalaria*, and possibly in *Navanax*, may be explained by lower competition between eggs than between spermatozoa⁶. *Hypoplectrus* have highly dispersed planktonic eggs³ with virtually no competition with eggs from the same parent, whereas spermatozoa compete among each other for the limited number of ovules available in each spawn (ovule parcelling⁵). In *Biomphalaria* and *Navanax* spermatozoa must compete for the limited number of ovules available in recipient snail, while young offspring of same mother readily disperse locally with no apparent competition for resources among them. In *Biomphalaria*, spermatozoa might also compete to fertilize ovules from the same parent in the case of selfing. Hence, sex allocation biased towards female function probably plays a role in the maintenance of sperm sharing in *Biomphalaria*². Given the capacity of these snails to store sperm for up to five months² and to digest it^{7,8}, it is possible that much of the sperm traded serves a nutritive function.

Thus, sperm sharing could be an evolutionary stable strategy in the presence of female function when there is bias towards female function which would enable the snail to trade sperm with other individuals while minimizing the risk of losing its own sperm.

Dr Paraense reports his unsuccessful trials on repeating sperm transference from donor to recipient through an intermediate snail. A mixture of recipient and

intermediary snails could not have occurred in our experiment because recipients were tagged with a red bead glued to the shell. This detail was not mentioned in the paper for two reasons: one, limitation on the space; the other is the irrelevance of the detail, because in planning to perform an experiment with two albino snails, the implication is that I could tell which was which. Furthermore, it is obvious that if intermediate and recipient snails were both laying wild-type hybrid eggs that had been mixed, the results still show that the recipient snail was laying wild-type eggs. As a matter of fact, Figure 2 shows that intermediary as well as recipient snails had laid wild-type hybrid eggs concomitantly, for at least one hundred successive days.

That we have mixed *B. tenagophila* and *B. occidentalis* is possible as nobody can identify the two species based on shell characters alone. As stated, our colleague Mara L. F. Dias and I are studying the occurrence of crosses between these two species, identifying them on the basis of reproductive organs as recommended in the paper where *B. occidentalis* was first described³. We only admit to dealing with *B. tenagophila* instead of *B. occidentalis* if the diagnostic characters for *B. occidentalis* are not actually diagnostic. In this case those characters must be reevaluated to identify properly the species *B. occidentalis*³. After all snails with *B. occidentalis* characters do crossbreed with snails with *B. tenagophila* characters in our laboratory.

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3. Fischer, E. A. *Am. Nat.* **117**, 64-82 (1981).
4. Maynard Smith, J. R. & Price, G. R. *Nature* **246**, 15-18 (1973).
5. Charnov, E. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2480-2484 (1979).
6. Bulmer, M. G. & Taylor, P. D. *Nature* **284**, 448-449 (1980).
7. Jong-Brink, M. Z. *Zellforsch.* **102**, 507-542 (1969).
8. Kitajima, E. W. & Paraense, W. L. *J. Morph.* **176**, 211-220 (1983).

The measurement of gross planktonic production

THERE are conflicting reports in the literature concerning the reliability of the ¹⁴C method, with indirect estimates of primary productivity differing from those provided by the ¹⁴C technique by factors of anywhere from 2 to 100 (ref. 1). The implications of such errors are far-reaching. Platt *et al.*² have pointed out that in some instances the reports have been based on comparison of methods of fundamentally different time scales and the comparison is therefore suspect, if not

invalid. From paired observation of *in vitro* oxygen- and ¹⁴C-derived measurements of production, we have concluded that we had no evidence for errors unique to the ¹⁴C technique in the measurement of gross primary production (GPP). We felt that this point had to be established with an oligotrophic population before undertaking direct comparisons of *in vitro* and *in situ* rates, which can be done with oxygen but not ¹⁴C. This would provide a chain of logic to enable us to determine whether there are in fact large errors associated with the ¹⁴C technique. No claim was made as to the accuracy of the *in vitro* techniques, although we did note that there was circumstantial evidence for their accuracy.

Smith *et al.*³ have argued that our approach did not have the necessary rigour to allow us to draw our conclusion. On the basis of a hypothetical food chain they argue that what they regard to be positive errors in the ¹⁴C technique could be compensated for by an overestimate of photosynthetic oxygen production, resulting from a rundown of respiration in the dark, the latter predicted from an empirical model. Even if the situation they envisage did exist in the samples we studied, the errors that the uncertainties would introduce (5-10%) are small in comparison with those we wished to consider. In the text we inadvertently used the ambiguous phrase "of any size". However, we presume it was evident from our introduction that our paper was concerned with errors of 2-100-fold (that is, the matter of the current debate over oceanic productivity measurements). Smith *et al.* also model unpublished data from a quite different location to illustrate how time-dependent changes might give rise to larger errors. The experimental protocol we used (in which we measured respiration rate after 0, 12, 24 and, in one instance, 36 hours of darkness and dissolved and particulate organic ¹⁴C measurement at the end of the light and the following dark period) was adopted to give warning of errors of this type. Thus we contend that our approach was adequate for the question we set out to answer and that the arguments of Smith *et al.* are not grounds for rejecting our conclusion.

Finally we must make it clear that it was not our wish to argue that the ¹⁴C technique gave an exact measurement of GPP. It has been recognized for over two decades that the compartmentation of tracer introduces uncertainties in the interpretation of ¹⁴C measurements. However, it is evident from our original paper that we share the views of Smith *et al.* and others (for instance ref. 4) that these types of errors, present in incubations *in vitro*, are not the prime cause of the large purported underestimates of GPP.

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1. Williams, P. J. leB., Heinemann, K. R., Marra, J. & Purdie, D. A. *Nature* **305**, 49–50 (1983).
2. Platt, T., Lewis, M. R. & Geider, R. in *Flows of Energy and Materials in Marine Ecosystems* (ed. Fasham, M. J. R.) 49–84 (Plenum, London, 1984).
3. Smith, R. E. H., Geider, R. & Platt, T. *Nature* **311**, 252–254 (1984).
4. Jackson, G. J. *Plankton Res.* **5**, 83–94 (1983).

SMITH ET AL. REPLY—The debate over the true level of oceanic primary productivity has tended to focus on possible shortcomings of the ^{14}C technique¹. A recurrent suggestion is that ^{14}C results are biased by the activity of heterotrophic organisms in the sample. The potential respiration of fixed tracer carbon by heterotrophic, as well as autotrophic, organisms makes it difficult to specify what level of production² is measured by ^{14}C and complicates comparisons against other means of estimating production. It seems less well recognized that methods other than ^{14}C can be similarly biased. We discussed³ the particular case of comparisons of O_2 with ^{14}C , using data from the literature for two different oceanic areas. We concluded that both ^{14}C and O_2 could underestimate GPP by factors ranging from near zero to at least twofold, depending on the trophic make-up and cycling rate of the communities, yet display excellent mutual agreement. The key to mutual agreement in the face of widely varying absolute bias and high community respiration rates was the metabolic dominance of microheterotrophs.

Williams and Marra wish to emphasize that their comparative measurements⁴ did at least refute the existence of errors much greater than twofold, unique to the ^{14}C technique, in the estimation of GPP in their samples. We would, and did, agree. Their original paper⁴ was not so clear on the scale of error in question. The abstract pointed specifically to the question of whether ^{14}C measures gross or net primary productivity, and it was concluded (p. 50) that “the technique measures gross rather than net production”. It appeared to be their contention that the measurements could discriminate gross from net primary productivity (in the sense of ref. 2, cited in ref. 4) but it is now explicitly agreed that they could not.

The experimental design of Williams *et al.*⁴ was admirably thorough and provided a number of tests for the larger bias predicted by our model for other oceanic areas. In fact, it was the availability of such tests in their data that allowed us to fit³ a model to the results and derive evidence for a metabolically dominant microheterotroph population. The model then allowed us to interpret observations on other oceanic areas to show that the trophodynamics of microplankton, and the magnitude of bias in production rates, was likely to differ considerably from that observed by Wil-

liams *et al.*⁴. An important point was that such differences would not be revealed by a simple comparison that treated the O_2 method as an absolute standard rather than as an operational production estimate. This conclusion was intended to be cautionary rather than to be critical of the particular findings of Williams *et al.*⁴.

Our model³ was extremely simple and of mainly heuristic value, but we should mention that Williams and Marra describe it incorrectly. The model entertains only negative errors, or underestimates, in measurements of GPP by both ^{14}C and O_2 . An erroneously low value for respiration in the O_2 dark bottle is predicted from the model, not from an empirical relationship, and such an error can cause only an underestimate of GPP, not an overestimate as Williams and Marra suggest. Loss of fixed tracer can cause only an underestimate of GPP by ^{14}C . Both mechanisms are recognized as sources of error in production estimates^{1,3} but only more recently have their quantitative implications been modelled^{3,5}.

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2. Strickland, J. D. H. *Bull. Fish. Res. Bd Can.* no. 122 (1960).
3. Smith, R. E. H., Geider, R. & Platt, T. *Nature* **311**, 252–254 (1984).
4. Williams, P. J. leB., Heinemann, K. R., Marra, J. & Purdie, D. A. *Nature* **305**, 49–50 (1983).
5. Jackson, G. A. J. *Plankton Res.* **5**, 83–94 (1983).

Hercynian orogeny in the Pyrenees was not a rifting event

THE Pyrenees were a segment of Hercynian crust that underwent rifting during the Mesozoic followed by compression during Late Cretaceous to Cenozoic time^{1–3}. From a regional study of Hercynian metamorphism in a North Pyrenean massif it was recently proposed⁴ that ‘the Hercynian orogeny in the Pyrenees was a rifting event... that affected a continuously subsiding Palaeozoic basin’. We believe that a rifting hypothesis for the Hercynian evolution of the Pyrenees is unlikely for the following reasons.

(1) The Pyrenees have suffered a strong pre-Permian and post-Carboniferous shortening resulting in polyphase deformation^{5–9}. This deformation is manifested by recumbent folding^{10–16}, nappe emplacement^{17–19} and subsequent steep axial-plane folding associated with strong slaty cleavage; Devonian and Car-

boniferous series are affected by these events^{20–22}. A late kinematic high-grade Hercynian metamorphism also developed in both the North Pyrenean zone and the Axial zone, where it affects the Devonian sequence^{7,15}.

(2) The Hercynian terrains of the Pyrenees represent only a small piece of a much larger Hercynian belt which extends across all of western Europe. The Hercynian is now considered to have been a typical collisional orogeny resulting from the closure of at least two oceanic domains²³. Taking into account the late Hercynian strike-slip faulting and the Mesozoic–Cenozoic displacement of Iberia relative to Europe along the North Pyrenean fault³, we conclude that the North Pyrenean massifs and the Axial zone of the Pyrenees are both external parts of the Hercynian belt. Thus the ‘schematic setting’ only related to rifting, proposed by Wickham and Oxburgh, is not in agreement with existing structural data in the Pyrenees, nor with the regional geology of western Europe.

Of course, a strong rifting did occur in the Pyrenees in association with strike-slip motion, but it began only in the early Cretaceous²⁴ and was accompanied by locally high-grade metamorphism²⁵, well known in the Mesozoic rocks. The Hercynian basement itself was affected by this Pyrenean event, as demonstrated by ^{40}Ar – ^{39}Ar dates in certain Pyrenean massifs, such as the St Barthélemy massif (H. Maluski, personal communication).

Contrary to the proposed rifting model⁴, the Hercynian metamorphism in the Pyrenees accompanies or just follows a strong pre-Permian, post-Carboniferous shortening observed everywhere in the Pyrenean basement. Thus it must be related to a thickening and not to a thinning of the crust.

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1. Mattauer, M. & Henry, J. *Geol. Soc. Lond. spec. Pub.* **4**, 3–23 (1974).
2. Choukroune, P. & Seguret, M. *Gravity Tectonics*, 141–156 (Wiley, New York, 1973).
3. Choukroune, P. & Mattauer, M. *Bull. Soc. géol. Fr.* **XX**, 689–700 (1978).
4. Wickham, S. M. & Oxburgh, E. R. *Nature* **318**, 330–333 (1985).
5. Bresson, A. *Bull. Soc. géol. Fr.* **14**, 1–278 (1903).
6. Mirouse, R. *Mém. Expl. Carte géol. Fr. Vol.* **7** 1–451 (1966).
7. Guiraud, G. *Mém. Bur. Rech. géol. min.* **63**, 1–349 (1970).
8. Santanach Prat, P. F. *Neues Jb. Geol. paläont. Abh.* **144**, 252–269 (1973).
9. Zwart, H. J. *Geol. Rdsch.* **53**, 170–205 (1963).
10. Seguret, M. & Proust, F. C. *r. hebdomadaire. Séanc. Acad. Sci., Paris* **266**, 317–320 (1968).
11. Matte, Ph. C. *r. hebdomadaire. Séanc. Acad. Sci., Paris* **268**, 1841–1844 (1969).
12. Matte, Ph. & Capdevila, R. C. *r. hebdomadaire. Séanc. Acad. Sci., Paris* **276**, 2867–2870 (1973).
13. Mattauer, M., Dalmayrac, B., Laubacher, G. & Vidal, J. C. *C. r. hebdomadaire. Séanc. Acad. Sci., Paris* **265**, 1361–1364 (1967).

14. Deramond, J. C. *r. hebd. Séanc. Acad. Sci., Paris* **272**, 693-696 (1971).
15. Zwart, H. J. *Leid. geol. Meded.* **50**, L, 1-74 (1979).
16. Majeste Menjoulas, Cl. *Bull. Soc. géol. Fr.* **XXIII**, 673-678 (1981).
17. Guitard, G. C. *r. hebd. Séanc. Acad. Sci., Paris* **258**, 4597-4599 (1964).
18. Autran, A. & Guitard, G. C. *r. hebd. Séanc. Acad. Sci., Paris* **269**, 2497-2499 (1969).
19. Bodin, J. & Ledru, P. C. *r. hebd. Séanc. Acad. Sci., Paris* **302**, 969-974 (1986).
20. Schmidt, H. *Abh. Ges. Wiss. Göttingen Math. Phys.* **8**, 1-85 (1931).
21. Heddebaut, Cl. *Bulletin* **IV**, 503 (1975).
22. Mirouse, R., Barrouquère, G., Bessière, G., Delvolvé, J. J. & Perret, M. F. *Geol. Rdsch.* **72**, 253-281 (1985).
23. Matte, Ph. *Tectonophysics* **126**, 329-375 (1986).
24. Albarède, F. & Michard-Vitrac, A. *Earth planet. Sci. Lett.* **40**, 327-332 (1978).
25. Ravier, J. *Mém. Soc. géol. Fr.* **38**, 1-200 (1957).

WICKHAM AND OXBURGH reply—Matte and Mattauer raise two objections to our interpretation of the Hercynian orogeny in the Pyrenees as a rifting event^{1,2,3}, the first relating to the Hercynian deformation style, the second to the regional tectonic interpretation of the entire Hercynian belt of Western Europe.

In the matter of Hercynian deformation style, we have no serious disagreement concerning many of the features described by Matte and Mattauer. We do not, however, believe that it is possible unambiguously to deduce the regional tectonic setting from such features. To the best of our knowledge no-one has a clear idea of the strain pattern expected in the middle crust during a strike-slip rifting event. We could certainly imagine heterogeneous deformation in such a setting that gave rise to features similar to those observed.

The second point raised by Matte and Mattauer is that rifting in the Pyrenees is not consistent with regional interpretations of the tectonic setting of the Hercynian of western Europe⁴. The Hercynian belt covers a very large area, and metamorphism and plutonism extended from the Devonian to the Permian^{5,6}. We do not believe that it is reasonable to ascribe a single tectonic mechanism to all of these events. Studies of recent orogenic belts have revealed considerable complexity in the spatial and temporal juxtaposition of different tectonic styles and thermal regimes⁷⁻¹¹. For example, the North Island of New Zealand contains an active rift zone characterized by high heat flow, hydrothermal systems and silicic magmatism^{8,10}. The crust of this region has many geological and geophysical similarities to the Hercynian crust of the Pyrenees¹⁻³. Yet this zone is located only 300 km west of an active subduction zone within which low-temperature, high-pressure metamorphism and compressional deformation are occurring.

It is unlikely that large-scale crustal thickening occurred in the Pyrenees during the Hercynian orogeny because late

Carboniferous uplift was not great. Post-orogenic Upper Carboniferous and Lower Permian red beds and volcanics commonly rest unconformably on marine Carboniferous and Devonian sediments, but never directly overlie high-grade basement rocks^{2,12}. This is in marked contrast with the large-scale uplift and deep erosion levels observed in modern collision belts, such as the Alps¹³ or the Himalayas¹⁴. Furthermore, there is a correlation between stratigraphic age, intensity of deformation and grade of metamorphism experienced by the Hercynian basement rocks in the Pyrenees. Upper Palaeozoic rocks have in general been only weakly deformed and metamorphosed, whereas Lower Palaeozoic metasediments experienced strong ductile deformation and amphibolite-facies metamorphism. This observation precludes the transport of near-surface material to deep structural levels in the crust, which is a common feature of overthrust terrains and collision belts.

The timing of the Hercynian metamorphism is fundamental to any tectonic interpretation. Age determinations on syn-metamorphic granites in the Pyrenees have yielded ages of 335 ± 15 Myr (whole-rock Rb-Sr on the "granite profond" at Canigou¹⁵) and 338 ± 5 Myr (ion-probe U-Pb on leucogranite zircon from the Trois Seigneurs massif; I. S. Williams, S. M. Wickham and W. Compston, in preparation). These Visean to Namurian ages are contemporary with Upper Carboniferous marine sedimentation documented throughout the entire Pyrenean region¹⁶. Metamorphism was contemporary with marine sedimentation at the surface. This situation is radically different from that expected in collision belts, where uplift precedes metamorphism by tens of millions of years^{13,17} but is entirely compatible with a rift setting^{1-3,18}.

The comments of Matte and Mattauer do not address two of the most remarkable features of the Hercynian Pyrenees, which we believe to be diagnostic of a rift setting. These are the exceptional thermal regimes which existed locally during metamorphism^{3,12,19}, and the deep hydrothermal systems which resulted in infiltration of the high-grade Palaeozoic metasediments by surface-derived marine fluids^{20,21}. Neither of these features is compatible with the known thermal and mechanical effects of crustal thickening⁹, but they are very similar to the observed geological and geophysical features of continental rift zones^{1,10,18,22-24}. A rift setting is further supported by the total absence of any high-pressure metamorphic rocks in the Pyrenees, by the lack of ophiolitic or oceanic rock types, and by the contemporaneity of marine sedimentation at the

surface and metamorphism at depth. We re-state our conclusion that Hercynian metamorphism in the Pyrenees occurred within localized rift zones, accompanied by intrusion of mantle-derived mafic material at depth. Rifting affected a deep, laterally extensive Palaeozoic sedimentary basin and promoted the low-pressure regional metamorphism, crustal anatexis and deep hydrothermal systems that characterize Hercynian metamorphic terrains in the Pyrenees.

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1. Wickham, S. M. & Oxburgh, E. R. *Nature* **318**, 330-333 (1985).
2. Wickham, S. M. & Oxburgh, E. R. in *The Geological Evolution of the Pyrenees* (eds Banda, G. & Wickham, S. M.) **A321**, 67-86 (1987).
3. Wickham, S. M. & Oxburgh, E. R. *Phil. Trans. R. Soc. A321*, 163-182 (1987).
4. Matte, Ph. *Tectonophysics* **126**, 329-375 (1986).
5. Bard, J.-P. *et al. Bull. geol. Rech. Min. Mém.* **107**, 161-189 (1980).
6. Autran, A. *et al. Bull. geol. Rech. Min. Mém.* **107**, 51-97 (1980).
7. Dallmeyer, R. D., Snoke, A. W. & McKee, E. H. *Tectonics* **5**, 931-954 (1986).
8. Stern, T. A. *Tectonophysics* **112**, 385-409 (1985).
9. Windley, B. F. *J. geol. Soc. Lond.* **140**, 849-865 (1983).
10. Walcott, R. I. *Phil. Trans. R. Soc. A321*, 67-86 (1987).
11. Weldon, R. & Humphreys, E. *Tectonics* **5**, 33-48 (1986).
12. Zwart, H. J. *Leid. geol. Meded.* **50**, 1-74 (1979).
13. Oxburgh, E. R. & England, P. C. *Ecol. geol. Helv.* **73**, 379-398 (1980).
14. Burg, J.-P. *Phil. Trans. R. Soc.* (in the press).
15. Vitrac-Michard, A. & Allègre, C. J. *Contr. Miner. Petrol.* **50**, 257-285 (1975).
16. Mirouse, R., Barrouquère, G., Bessière, G., Devolvé, J. J. & Perret, M. F. *Geol. Rdsch.* **72**, 253-281 (1985).
17. England, P. C. & Thompson, A. B. *J. Petrol.* **25**, 894-928 (1984).
18. Sandiford, M. & Powell, R. *Earth planet. sci. Lett.* **79**, 151-158 (1986).
19. Wickham, S. M. *J. Petrol.* (in the press).
20. Wickham, S. M. & Taylor, H. P. *Jr. Contr. Miner. Petrol.* **91**, 122-137 (1985).
21. Wickham, S. M. & Taylor, H. P. *Jr. Contr. Miner. Petrol.* (in the press).
22. Baldrige, W. S., Olsen, K. H. & Callender, J. F. *New Mexico Geological Society Guidebook, 35th Field Conference, Rio Grande Rift*; 1-12 (Northern, New Mexico, 1984).
23. Lachenbruch, A. H., Sass, J. H. & Galanis, S. P. *Jr. J. geophys. Res.* **90**, 6709-6736 (1985).
24. Taylor, H. P. *Jr. in Hydrothermal Processes at Seafloor Spreading Centres* (eds Rona, P. A., Bostrum, K., Laubier, I. & Smith, K. L.) **83**-139 (Plenum, New York, 1983).

Matters Arising

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Flow cytometry in biomedical science

James V. Watson

LS2

Lasers have significant applications in biology, as recent advances in flow cytometry demonstrate.

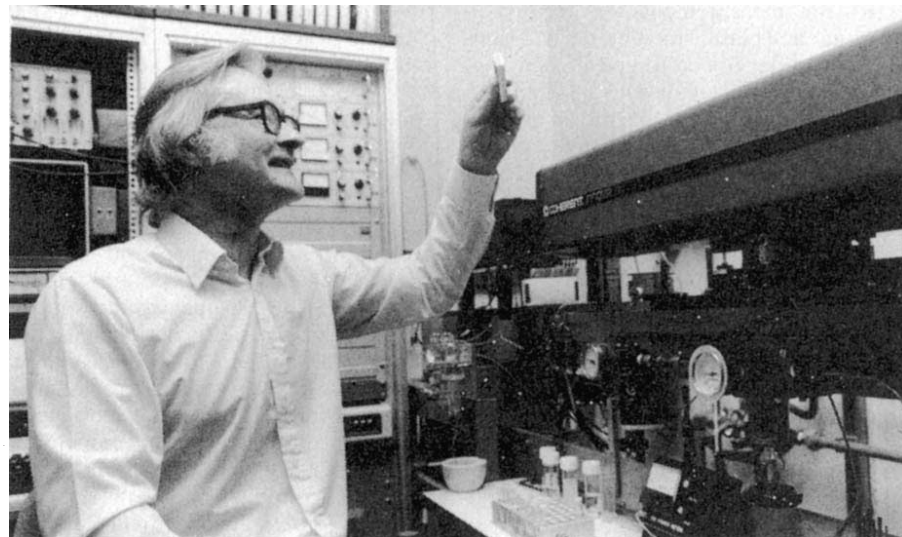
Flow cytometry (FCM) is being used increasingly in all branches of biological and medical science due to its quantitative capacity, versatility, sensitivity, speed, statistical precision, and ability to identify cell subsets in, and sort them from, heterogeneous populations. Quantitation of FCM relies upon both fluorescence and light scatter measurements from individual cells, and the power of the technique derives from the ability to make these multiple measurements of each cell simultaneously, at very rapid rates. Typically, up to 5,000 cells can be analysed per second and sorting rates of 3,000 to 4,000 cells per second with greater than 90 per cent purity can be achieved.

Streams of cells

A single cell suspension is streamed coaxially through a flow chamber so that individual cells pass single file through the focus of a high intensity excitation light source. Lasers are frequently used because the laser light beam is very stable and easily focused to small volumes, and the high light flux excites sufficient fluorescence from individual cells to be measured in the few microseconds taken to traverse the focal volume. Each flash of scattered light emitted from a cell, or fluorescence from cells containing suitably stained molecules, is picked up by a light collection system and filtered sequentially by dichroic mirrors. These reflect specific wavelengths into photomultipliers (PMTs) which are additionally 'guarded' by band pass filters. The quantity of light incident on each PMT, responding within its given colour band, is then proportionally converted to an electrical signal. This is amplified, digitized, stored transiently in computer memory and thence stored on disk for subsequent display and analysis.

Many commercial machines are now equipped with four detectors and two lasers, for two- or three-colour fluorescence work. However, some multi-laser research and development instruments have up to eight detectors¹ and one such instrument has the ability to collect data on 32 light scatter detectors simultaneously². Data processing and display procedures have been developed which handle up to 8-dimensional fully cross-correlated data in a single pass through the data set, using microcomputers with only 28K addressable memory¹.

Cell sorters originate from developments in ink jet writing³. These instruments deflect electrostatically charged



James V. Watson seated before the Cambridge flow cytometer, the prototype for most current flow cytometers.

droplets, each of which contains a single cell with given fluorescence and light scatter characteristics, into collection vessels for subsequent identification or biological manipulation.

Staining techniques

The applications of this technology are legion and are increasing as the sensitivity increases and as new probes are developed. A detection limit equivalent to 150 molecules of free fluorescein per cell has been achieved⁴, and with innovations currently under development this could be further improved by a factor of five to ten. Fluorescent dyes and staining techniques fall into three categories: those in which the ligand itself is fluorescent and is accumulated within the cell, those that require interaction of the ligand with a cellular constituent to release the fluorophore or enhance its emission, and fluorochrome-coupled antibodies.

Nucleic acid dyes have been used extensively in FCM and include DNA- or nucleic acid-specific ligands and polyanion dyes such as acridine orange, for both monovariate and bivariate cell cycle analysis. Acridine orange has the unusual property of giving rise to green fluorescence from double-stranded nucleic acids and red fluorescence from single strands, and selective denaturation of double-stranded RNA gives a simultaneous quantitation of both nucleic acids⁵. This dye has also been used in a cervical cancer prescreening programme in conjunction with slit scanning to give nuclear/cytoplasmic ratios. Blind trials of the technique ex-

bited a false negative rate of 0.18 per cent and about a 10 per cent false positive rate⁶.

The bis-benzimidazoles bind in the minor groove of the DNA molecule, and have interesting properties because their emission spectrum when bound to DNA is dependent on the DNA:dye ratio and on the pH of the microenvironment. At low dye:DNA ratios the fluorescence emission is short wavelength (violet)-biased but at higher ratios the emission is long wavelength (green)-biased⁷. These differences can be measured by using two or more detectors responding to different regions of the emission spectrum⁸. This phenomenon has enabled different subsets to be identified within heterogeneous populations, enabling the identification of cells resistant to cytotoxic agents, which excrete dye molecules by the same mechanisms they use to excrete the drug. Hence, cells resistant to the cytotoxic agent exhibit a violet-biased Hoescht 33342 emission spectrum, while cells that succumb to the cytotoxins display a longer wavelength-biased emission spectrum.

Nuclei can be extracted from archival human biopsies for DNA ploidy studies using a number of fluorochromes, including DAPI and ethidium bromide⁹. Such studies can reveal aneuploid components, yielding information on which to base future prognoses¹⁰. These techniques have been extended to include the simultaneous assay of DNA and nuclear-associated oncogene-encoded proteins¹¹ which may be clinically useful for cervical cancer prescreening¹². The simultaneous determination of total versus newly repli-

cated DNA can also be assayed with similar types of techniques using anti-BrdU antibodies¹³ or biotinylated nucleotides¹⁴. DNA fluorochromes are also used for chromosome analysis with both single¹⁵ and dual laser¹⁶ systems, not only for automated karyotyping, but also for constructing cloned gene libraries from individually sorted chromosomes¹⁷.

Kinetic measurements of both cytoplasmic¹⁸ and plasma membrane¹⁹ enzymes have been developed to give K_m and V_{max} for reactions in populations of viable intact cells, using fluorogenic substrates based on fluorescein and methyl-umbelliferones. These methods have now been extended to determine the level of reaction inhibition induced by carbamylating cytotoxic agents. This is an indirect method for assaying the transport of the agent across the external membrane, and for determining its cytotoxic efficacy once it enters the cell²⁰.

Fluorophore-coupled monoclonal antibodies have been used in conjunction with FCM in a bewildering number of studies in both cell biology and medicine. Perhaps the most widely studied are the haemopoietic and related systems. Automated leucocyte typing in leukaemias, immunosuppressed transplant patients and lymphomas are now routine in many hospitals. The rapid progress in elucidating details of bone marrow lineages²¹ over the last five years has been due to the development of suitable monoclonal antibodies, and to the refinement of cell sorting flow cytometry techniques. □

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1. Watson, J.V., Horsnell, T.S. & Smith, P.J. *Cytometry* (in the press).
2. Salzmann, G.G., Crowell, J.M., Goad, C.A., Mullaney, P.F. & Coulter, J.R. *Clin. Chem.* **21**, 1297-1304 (1975).
3. Sweet, R.G. *Rev. Sci. Instr.* **36**, 131 (1965).
4. Watson, J.V. & Walport, M.J. *J. immun. Meth.* **93**, 171-175 (1986).
5. Traganos, F., Darzynkiewicz, Z., Sharpless, T. & Melamed, M.R. *J. histochem. Cytochem.* **25**, 46-56 (1977).
6. Wheelless, L.L. *et al. Cytometry* **5**, 1-8 (1984).
7. Smith, P.J., Nakeff, A. & Watson, J.V. *Exp. Cell Res.* **159**, 37-46 (1985).
8. Watson, J.V., Nakeff, A., Chambers, S.H. & Smith, P.J. *Cytometry* **6**, 310-315 (1985).
9. Hedley, D.W., Friedlander, M.I., Taylor, I.W., Rugg, C.A. & Musgrove, E.A. *J. histochem. Cytochem.* **31**, 1333-1335 (1983).
10. Friedlander, M.I., Hedley, D.W. & Taylor, I.W. *J. clin. Path.* **37**, 961-974 (1984).
11. Watson, J.V., Sikora, E.K. & Evan, G.I. *J. immun. Meth.* **83**, 179-192 (1985).
12. Hendy-Ibbs, P., Cox, H., Evans, G.I. & Watson, J.V. *Br. J. Cancer* (in the press).
13. Gratzner, H.G. *Science* **218**, 474-475 (1982).
14. Blow, J. & Watson, J.V. *Cell* (in the press).
15. Sillar, R. & Young, B.D. *J. histochem. Cytochem.* **29**, 74-78 (1981).
16. Langlois, R.G., Yu, L.C., Gray, J.W. & Carrano, A.V. *Proc. natn. Acad. Sci. U.S.A.* **79**, 7867-7880 (1982).
17. Davies, K.E., Young, B.D., Elles, R.G., Hill, M.E. & Williamson, R. *Nature* **293**, 374-376 (1981).
18. Watson, J.V., Chambers, S.H., Workman, P. & Horsnell, T.S. *FEBS Lett.* **81**, 397 (1977).
19. Watson, J.V., Workman, P. & Chambers, S.H. *Biochem. Pharmacol.* **28**, 821-829 (1979).
20. Dive, C., Workman, P. & Watson, J.V. *Cytometry* (in the press).
21. Watt, S.M. *et al. Molec. biol. Med.* **2**, 351-368 (1984).

The latest in lasers

This week features new developments in lasers and their accessories, plus a review of laser-based instruments for biological applications.

COHERENT has a wide range of **small frame ion lasers** for a variety of applications (Reader Service No. 100). The Innova 90 has a metal ceramic tube design, and is now available with a 6 W specification, completing the existing Innova 90 range which includes 3, 4 and 5 W argon systems and a krypton version. The £28,600 (UK) Innova 90 has a series regulated power supply, a remote control module, and a sealed intracavity space, and has now been expanded to incorporate a longer plasma tube, and a super invar resonator. The Innova 70 series is designed for high performance in the 2 to 4 W range at low cost, priced at around £12,500 (UK). Other features include light and current control, aperture wheel, compensated wavelength selector and all-line mirror mount. The Innova 70 comes as a package with a dye laser and power meter.

The new Microyag-1 **diode-pumped Nd:YAG laser** from Photon Control Ltd combines the best of both laser types (Reader Service No. 101). The low-



Combines the best of two laser types.

divergence beam of the Microyag-1 has an output wavelength of either 1.06 or 1.3 μm , and the Microyag-1 has continuous wave, gain switched, pulsed and optional Q-switched operating modes. Costing £8,400-£14,400 (UK) Photon Control's new laser has applications in fibre optic sensor research, coherent communications, injection locking and inspection and testing.

Spectra-Physics has extended its line of **dye lasers**. The Model 355 ion-mountable dye laser combines with the Model 2020, 2025, or 168 argon ion lasers to form a compact dye laser system (Reader Service No. 102). The £10,000 (UK) Model 355 uses a choice of two dyes,

rhodamine 6G or kiton red, with maxima of 595 and 640 nm, respectively, for a tuning range of more than 40 nm for each dye. Specified power exceeds 750 mW at the peak of each dye. The two-mirrors of the Model 355 produce high conversion efficiency and high spectral purity, according to Spectra-Physics, and the birefringent filter is set in the cavity at Brewster's angle for minimal loss.

Pulse Systems, Inc. makes **CO₂ lasers** with full-width, half-maximum pulse widths of approximately 75 μs (Reader Service No. 103). The pulse shape can be

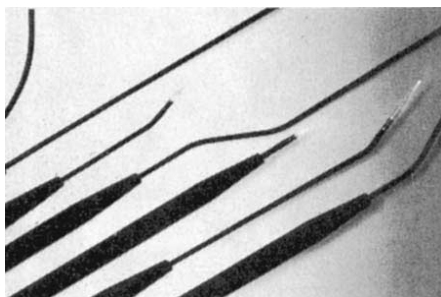


A CO₂ laser that doesn't require a helium supply and is insensitive to O₂.

varied from 10 ns to >200 μs using other main-pulse discharge circuits and/or by adding Q-switching and cavity dumping to the laser cavity. The \$3,498-\$19,000 (US) unit is complete, with all power supplies, pulse electronics and internal-trigger system included. Pulse System's lasers can operate without a helium supply, using a 50-50 mixture of CO₂ and air, and are therefore insensitive to O₂.

The **pulsed Nd:YAG lasers** available from Laser Photonics are geared toward research applications (Reader Service No. 104). The modular design of Laser Photonics' products allow it to accommodate most customized laser needs upon request. The unique design of Laser Photonics' transmitter module employs a folded-beam resonator, a configuration that eliminates problems caused by misalignment of mirrors due to shock, vibration and thermal stress. Closed cycle cooling means no outside water supply is needed for operation, and a simmered flash lamp supply achieves pulse-to-pulse stability. Laser Photonics' line of Nd:YAG lasers range in price from \$15,000 to \$25,000 (US).

Lasers are making a name for themselves in the surgical theatre, and Surgical Laser Technologies, Inc. is providing **contact lasers** to lead the way (Reader Service

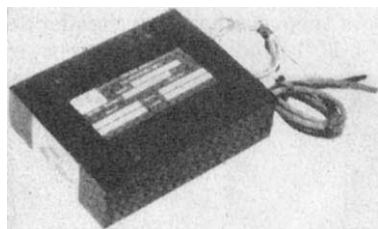


Surgical Laser Technologies' laser tips take accurate aim in laser surgery.

No. 105). Its \$65,000 (US) contact laser combines the attributes of CO₂ lasers for cutting, YAG lasers for coagulating, and argon lasers for subsurface coagulation into one portable unit. Surgeons change the sapphire tip to enable them to perform these operations with little preparation. The low power operation of the contact lasers means no uncontrollable laser energy backscatter that can damage healthy tissue around the incision.

Accessories for lasers

Power Technology, Inc. makes **power supplies** to keep lasers running (Reader Service No. 106). The Series L23 laser power supplies offered by Power Technology are compact, solid state, regulated power supplies designed for use with



The power supply for helium neon lasers.

helium neon laser tubes. Many shapes and sizes are available to fit a large variety of packages. Switching mode regulation provides high efficiency, reducing the need for a heat sink, thus increasing the life of the laser tube seals and other heat sensitive components. The \$50–100 (US) L23 series is suitable for virtually all 12V battery powered applications, according to Power Technology, and also for many a.c. line applications where 12 to 15V d.c. is available from a bulk power supply. Also for helium neon lasers, the L92 series is a current regulated, switching mode, dual input voltage power supply for operation from a.c. input voltages of 100–130V or 200–260V. The \$60–110 (US) L92 is fully potted in black fibreglass casing for protection from dust and moisture.

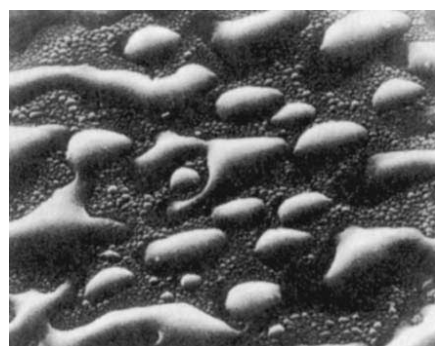
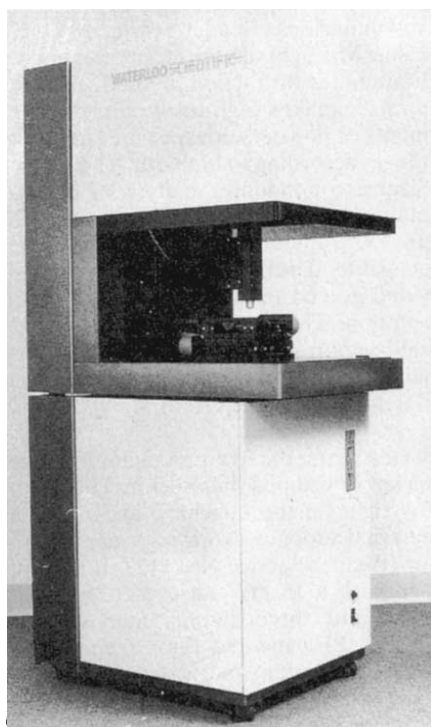
If **monitoring actual power level** during operation of high energy infrared lasers is a concern, Oriel Scientific Ltd offers the LIS-4000 Infrared Integrating Sphere

(Reader Service No. 107). The 4-inch diameter water-cooled sphere has a gold coating, optimized for diffuse reflectance at the CO₂ laser wavelength, and can be used in conjunction with almost any infrared detector that mounts at a port on the integrating sphere. For monitoring during operation, the LIS-4000 can be mounted at the back mirror of the laser cavity. Oriel's £1,976 (UK) Infrared Integrating Sphere can also be supplied with a coating optimized at other infrared laser lines as well.

For real-time, high-resolution **images of infrared laser beams**, Oriel Scientific Ltd distributes Optical Engineering's water-cooled thermal image plates (Reader Service No. 108). According to Oriel, the water-cooled design allows continuous viewing of laser beams of up to 500 W. The fast response time permits viewing of rapidly changing laser mode patterns when adjusting resonator mirrors. The 5 × 5 inch plates come in four sensitivity ranges contained on two plates, and cost £236 (UK).

Laser-based instruments

The Waterloo Scientific WSI-100 **scanning laser microscope** uses a red HeNe laser beam focused to a diffraction-limited spot for optical beam-induced current measurements in semiconductors, solar cell and photovoltaic device testing, and automated testing of thin film devices and arrays (Reader Service No. 109). With the WSI-100, all functions are computer-controlled, as the specimen is moved in a raster scan on precision translation stages under a fixed laser beam. The resulting



At first glance appearing like water beading up on a waxed surface, this scanning electron micrograph actually reveals the result of piercing a silicon wafer with a laser. The laser beam drilled a hole by heating and almost instantly vapourizing the silicon, that subsequently condensed and resolidified on the surface of the wafer as tiny drops. This photo is one of 50 photomicrographs, photomacrographs, and electron microscopic images comprising the exhibit "MicroScapes: The Hidden Art of High Technology", on display at the US National Academy of Sciences until 12 March. Produced and developed by AT&T to display advanced developments in microelectronics and lightwave communications, "MicroScapes" represents some of the current processes used in the production of advanced communications systems. The exhibit includes micrographs of metal crystals, cross sections of preforms from which optic fibres called lightguide are drawn, and close-ups of microprocessor chips that reveal the complex configuration of circuits. Noteworthy among the collection is a scanning tunnelling micrograph of atoms on a silicon surface, the first glimpse of an atom gained via the Nobel-prize winning technology. The exhibition is on a three-year tour of the United States under the auspices of the Association of Science-Technology Centers in Washington, D.C., and will constantly be updated during its run — a reflection of the rapid pace in which new developments are being made in telecommunications.

image is displayed on a high-resolution colour monitor, in false colour, gray scale, or Y-deflection line images (with or without hidden line eliminator). Image enhancement and analysis software is also included in the WSI-100 scanning laser microscope's \$90,000 (US) price.

From Wyatt Technology Corp. comes a **high performance laser chromatography system** for gel permeation chromatography (GPC) (Reader Service No. 110). The \$18,500 (US) DAWN GPC Model F multi-angle laserlight scattering detector allows near real time determination of both absolute molecular weights and molecular sizes without calibration of the GPC column. Its low-volume flow cell allows low- and high-angle scattering measurements

to be made instantly. The DAWN GPC contains four optical fibre-coupled photomultipliers placed at angular locations specified by the user, as well as 14 high-gain hybrid photodiode detectors spanning an angular range from about 10° to 160° . A 5 mW polarized HeNe laser is used as the light source, although tunable argon-ion and helium-cadmium lasers of 10–20 mW power options can be provided for increased sensitivity. The DAWN system may be connected to a standard concentration-sensitive detector, using its associated GPC software package written for IBM XT/AT computers. The software collects data continuously from the DAWN and the concentration detector for the duration of the chromatographic run, and calculates and graphs solvent baseline determinations as well as peak widths, heights and delay times.

Spectrolab Ltd combines a pulsed laser, a micro-zoom optic and an emission spectrometer to come up with its Laser Emission

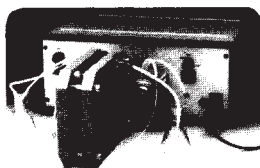


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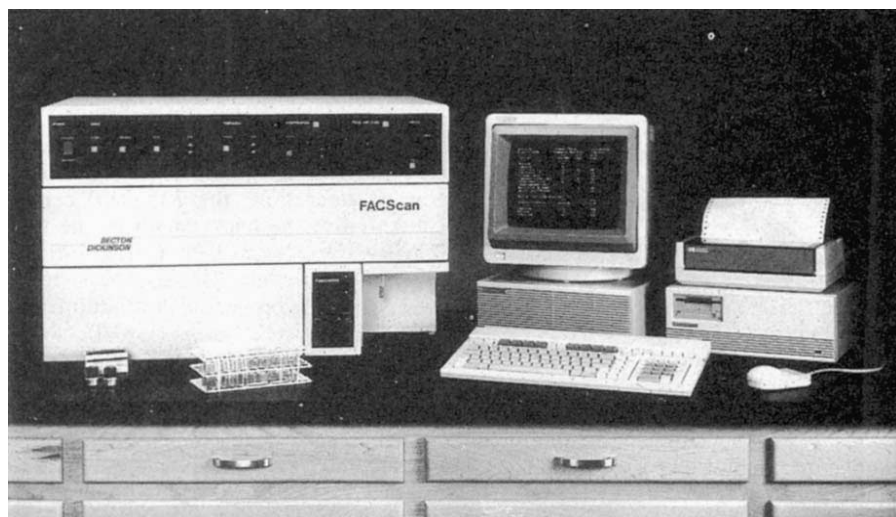


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Reader Service No.47



FACScan: Becton Dickinson's complete system for flow cytometry.

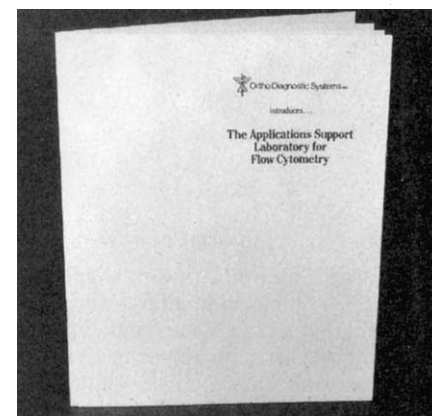
sion Microprobe, an instrument that succeeds in obtaining **atomic emission analysis** of microscopic-sized samples (Reader Service No. 111). Spectrolab's \$35,000 (US) Laser Emission Microprobe uses a pulse of light from a laser, focussed onto an area of the sample no more than a few micrometres in diameter. The laser beam vapourizes the illuminated area, and the vapourized material triggers an electric arc between two electrodes, causing an emission of radiation that characterizes the sample. Spectrolab claims the technique is so sensitive that a complete atomic analysis may be performed on samples only 20 μm in cross-section in just a few nanoseconds.

Malvern takes care of **particle sizing** needs with its MasterSizer, a laser-based particle size analyser for powders, sprays and emulsions (Reader Service No. 112). Using Mie light scattering analysis and the Fraunhofer diffraction method, the MasterSizer makes high-resolution measurements of powders down to the submicron range, according to Malvern. The MasterSizer accommodates analysis of particles in two size ranges: 0.1–80 μm and 1.2–600 μm . A third range, 0.5–170 μm , is also available. Each range is further subdivided into 64 size classes to maximize resolution. The MasterSizer has automatic calibration and detector alignment, and all operations can be automated by computer control, if required.

FACScan, the five-parameter **flow cytometer** developed by Becton Dickinson, fits right on the benchtop and needs no external supplies of water or compressed air (Reader Service No. 113). It is equipped with a 15 mW air-cooled argon-ion laser and three fluorescence channels (FITC, PE, and red fluorescence), plus forward scatter and side scatter. The \$74,900 (US) FACScan comes with a Consort 30 computer workstation complete with monochrome monitor, allowing

automated monitoring of sample flow and laser power, and analysis using menu-driven applications software for lymphocyte phenotyping, immune monitoring, DNA analysis, reticulocyte counting and platelet studies. Accessories for the FACScan include the FACS AutomATE for sample introduction, and colour graphics.

To back up its line of flow cytometers, Ortho Diagnostic Systems is offering enrollment in its free **flow cytometry applications support** laboratory (Reader Service No. 114). Under the applications support



Outlines the details of Ortho Diagnostic's application support for flow cytometry programme.

programme, enrollees will have access to the laboratory's telephone consultation service for questions concerning flow cytometry, reagents or applications. Participants will also receive a complete bound set of Ortho Diagnostic Systems' *Protocols for Flow Cytometry*, a set of 15 step-by-step applications for flow cytometry in DNA analysis, cell viability testing, and differential cell analysis. □

These notes are compiled by Carol Ezzell from information provided by the manufacturers. To obtain further details about these products, use the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.